STUDIES ON HUMAN ALPHA UTERINE PROTEIN

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"Beware that you do not lose the substance by grasping at the shadow ."

Aesop c.550 B.C.

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Summary

The aim of this research was to further the existing information on the human protein known as alpha uterine protein (AUP). Several independent lines of research were pursued and the following results were reported:

1. AUP from two of the major sources known, namely uterine decidua and amniotic fluid, was found to be immunologically- and electrophoretically-identical. This confirms a previous report of identity between AUP from these two sources. In addition, the molecular weight of amniotic fluid AUP was estimated by gel filtration to be approximately 53,700 daltons, which is similar to that previously estimated for decidual AUP by the same method.

2. An unusual peak morphology of AUP on AACE was observed and investigated. The area enclosed by the AUP precipitin peaks was more deeply-staining than the surrounding gel, resulting in a "filled-in" appearance. This was found to be a feature of antibody/antigen specificity in that it was observed with antisera raised against AUP of decidual origin but not with antisera raised against amniotic fluid AUP. The exact nature of this effect remains uncertain. However, it may indicate incomplete antigenic identity between AUP from amniotic fluid and decidua, thereby contradicting the existing evidence of identity between AUP from these two sources, described in 1.

3. A component which may be structurally-related to AUP was detected in term cord serum. This was found to be of fetal rather than maternal origin. However, there was no evidence of immunological cross-reactivity between this "AUP-like" component in TCS and AUP.

4. To date, AUP has been detected in endometrium, myometrium, early gestation decidua, amnion, chorion and amniotic fluid. In this study, several other sources of AUP were discovered, namely early gestation (8-13 weeks) and term placenta, term decidua and mid-gestation fetal gut. Placental AUP was found to be electrophoretically- and immunologically-identical to that present in amniotic fluid and decidua.

5. The levels of AUP in early gestation (8-13 weeks) and term decidual and placental tissue were measured by one-dimensional AACE. Contrary to existing data on amniotic fluid AUP levels, no correlation between gestational age and concentration was found in either decidua or placenta. Similar levels of AUP were detected in both these tissues early in gestation, although the concentration in decidua was slightly higher than that in placenta at each stage of gestation. An overall decrease in AUP concentration towards term was observed in the decidua and placenta. However, the decline in concentration was much greater in placental tissue and only trace amounts of AUP were detectable at term.

6. An attempt to purify AUP from amniotic fluid by the three-step method of antibody affinity chromatography, ion exchange chromatography and gel filtration previously used to purify AUP from 11 week gestation decidua was unsuccessful. However, a two-step procedure involving ion exchange chromatography and Concanavalin-A Sepharose chromatography gave better results. The resultant partially-purified material was enriched with AUP by approximately 21-fold and the concentration of the major contaminant protein, serum albumin, was reduced to about $\frac{1}{12}$ of the initial level. However, no further purification was performed due to the relatively low yield of AUP obtained from the Con-A Sepharose chromatography step. The partially-purified AUP was radioiodinated

with ^{125}I and immune precipitates were analysed by SDS-polyacrylamide gel electrophoresis under denaturing conditions. Three components of molecular weights 48000, 16000 and 9000, respectively, were observed. This may indicate that amniotic fluid AUP is structurally different from that present in decidual tissue. However this observation awaits further confirmation.

7. The origin of AUP in amniotic fluid was investigated by comparing levels of this protein in samples of 2nd trimester amniotic fluid with levels of several serum proteins and two non-serum proteins; α1,4-glucosidase and prolactin. The concentrations of the latter two proteins between 10 weeks of gestation and term vary in a similar manner to the concentration of AUP. Hence a common origin in amniotic fluid for these three proteins is likely. In contrast, the concentration profile of the major serum proteins in amniotic fluid is markedly different during this period of gestation. A positive correlation was found between levels of albumin, Gc and α_1-antitrypsin in the amniotic fluid samples, which supports other evidence that these proteins have a common maternal serum origin. In contrast, no correlation was found between levels of AUP and either prolactin or α1,4-glucosidase. This suggests that the origin of AUP and α1,4-glucosidase in amniotic fluid may differ. In the case of prolactin, however, no conclusions can be made because the assay results were not included in the final statistical analysis due to a non-linearity. Therefore a common origin in amniotic fluid for AUP and prolactin cannot be discounted.

8. Immunological identity was found between AUP and progestagen-dependent endometrial protein (PEP).

9. A progesterone-binding study was carried out using the method of equilibrium dialysis to investigate the possibility that AUP functions as a progesterone-receptor in vivo. However, no evidence of specific binding of progesterone to AUP was found.

List of Abbreviations

AUP	:	Alpha uterine protein
PEP	:	Progestagen-dependent endometrial protein
α_1-AT	:	Alpha$_1$-antitrypsin
Gc	:	Group-specific component
BSA	:	Bovine serum albumin
PDH	:	Pyruvate dehydrogenase
AF	:	Amniotic fluid
TCS	:	Term cord serum
MS	:	Maternal serum
TEMED	:	NNN'N'-Tetramethylethylene-diamine
Con-A	:	Concanavalin-A
PBS	:	Phosphate-buffered saline (0.16M NaCl + 5 mM potassium phosphate, pH 7.5)
1D AACE	:	One-dimensional antibody-antigen crossed electrophoresis
2D AACE	:	Two-dimensional antibody-antigen crossed electrophoresis
O.D.	:	Optical density

INTRODUCTION

Introduction

The scope of research on amniotic fluid proteins

In recent years there has been a marked increase in research on the proteins in amniotic fluid. In particular, there has been considerable interest in the search for fetal- and pregnancy-specific proteins. This follows the discovery of an association between raised levels of alphafetoprotein (AFP) and fetal neural tube defects, especially anencephaly and spina bifida (Brock and Sutcliffe, 1972). Alphafetoprotein is a fetal serum protein synthesised in fetal liver and yolk sac (Gitlin and Boesman, 1967; Gitlin and Perricelli, 1970). There is evidence that the yolk sac begins synthesising AFP by the 6th week of gestation, whereas the liver commences synthesis even earlier at around 4 weeks of gestation (Gitlin and Biasucci, 1969; Gitlin and Perricelli, 1970). By the end of the frist trimester, the yolk sac has degenerated and become atretic, therefore no longer contributes to the fetal serum AFP pool. The liver, however, continues to synthesise AFP throughout gestation, with maximum concentrations reached in fetal serum around 13 weeks of gestation. Although the total amount of serum AFP synthesised continues to increase until 20 weeks' gestation, the concentration in serum decreases after the 13th week because the fetal growth rate exceeds the rate of AFP synthesis during this period (Gitlin and Boesman, 1967). Fetuses affected with neural tube defects have normal AFP levels (Brock, 1974; Seller *et al*, 1974; Seppälä and Ruoslahti, 1974), therefore the increased levels in the surrounding amniotic fluid are thought to be due to either leakage of fetal cerebrospinal fluid or to transudation of AFP from fetal capillaries in contact with the amniotic fluid.

Elevated levels of AFP in amniotic fluid have also been linked with other fetal disorders e.g. congenital nephrotic syndrome (Seppala et al, 1967) and exomphalos (de Bruijn and Huisjes, 1975; Nevin and Armstrong, 1975; Seppala et al, 1976), although in the latter case at least 50% of affected fetuses have other malformations, including anencephaly (McKeown et al, 1953).

There is also evidence of an association between abnormal levels of some enzymes in amniotic fluid and inborn errors of metabolism. For measurements of enzyme activities in amniotic fluid to be of diagnostic value, the enzyme in question must be shown to be derived exclusively from the fetus. This has proved to be a major problem in such studies. For example, Tay-Sachs disease and Hunter's syndrome are genetic disorders which are characterised by the deficiency of the enzymes hexosaminidase A and iduronate sulphatase, respectively, in the tissues of the affected fetus. There is a parallel deficiency of these enzymes in the amniotic fluid, and this has been used to identify these disorders in utero (Schneck et al, 1970; O'Brien et al, 1971; Friedland et al, 1971; Navon and Padeh, 1971; Liebaers et al, 1977). In some cases of both Tay-Sachs disease and Hunter's syndrome, however, residual enzyme activity has been detected in the amniotic fluid. This indicates an additional source of these enzymes in the fluid, possibly maternal (Friedland et al, 1971; Liebaers et al, 1977; Geiger et al, 1978), and raises doubts about the reliability of these assays for prenatal diagnosis. Other factors which present problems when assaying enzymes associated with genetic disorders include the possibility of blood contamination of samples, the wide variability of "normal" levels of enzymes in amniotic fluid (Sutcliffe, 1981), and the possible presence of fetal isoenzymes not affected by the disease

(Burton et al, 1974). It is therefore apparent that further investigation as to the nature and origin of the enzymes present in amniotic fluid, and the exact effect(s) of the genetic disorder in question is required in order to determine the usefulness of enzyme studies in prenatal diagnoses.

Measurement of levels of fetal-specific proteins present in amniotic fluid during gestation may also serve as an index of fetal maturity. The estimation of fetal lung maturity is of particular importance because it is used to predict respiratory distress syndrome in newborn infants. The amount of phospholipids present in amniotic fluid, especially the ratio of lecithin to sphingomyelin (Gluck et al, 1971) is used to assess fetal lung development. This test is based on the rationale that the phospholipids, especially lecithin, present in the amniotic fluid originate principally from the fetal lungs. A great deal of indirect evidence supports this hypothesis, e.g. the fatty acid composition of amniotic fluid and pulmonary lecithins are similar, but differ from those of the lecithins present in fetal skin, membranes, saliva, urine, placenta and maternal blood (Gluck et al, 1971). In addition, several studies have indicated the direct passage of fluid from the fetal lungs into the amniotic cavity (Setnikar et al, 1959; McLain, 1973; Gluck et al, 1974). However, there is as yet no unequivocal evidence that pulmonary phospholipids enter the amniotic fluid. Condorelli et al (1974) investigated the possibility of extra-pulmonary sources of amniotic fluid phospholipids by injecting ^{14}C-palmitate intravenously into three sheep fetuses at or near term, and examining the isotopic distribution in the lipids of pulmonary fluid, amniotic fluid, allantoic fluid, fetal serum and urine. The sampling

method used effectively isolated the fetal pulmonary fluid compartment from the mouth, gastrointestinal tract and amniotic fluid. Despite this, radiolabelled phospholipids were present in all the fluids examined, including amniotic fluid. Thus, it is possible that there are extrapulmonary sources of amniotic fluid phospholipids. On the basis of both quantitative and qualitative examinations of the phospholipids in the various compartments, the authors suggested that fetal serum and/or urine may contribute to a major extent to amniotic fluid phospholipids. However, since the pulmonary compartment was isolated in this experiment, a contribution from the fetal lungs cannot be excluded. If the extrapulmonary contribution proves to be greater than that from the fetal lungs then the phospholipid test would reflect pulmonary maturation as a function of the general development of the fetus, rather than lung maturity itself. Thus, alternative markers of fetal lung development may be sought in the future.

One such marker which may prove useful in the assessment of fetal lung maturity is pulmonary apoprotein which is present in amniotic fluid (King et al, 1975). This apoprotein, which was first isolated from adult human pulmonary surfactant, consists of three components of molecular weights 69000 (identified as albumin), 34000 and 11000, respectively. It is possible that the 34000 dalton component is identical to the pulmonary glycoprotein of similar molecular size reported in the alveoli of patients with alveolar proteinosis and lung lavage material of normal animals (Bhattacharyya et al, 1975, 1976a,b; Bhattacharyya and Lynn, 1977a,b), and in human amniotic fluid (Bhattacharyya and Lynn, 1978). Using an antiserum raised against adult surfactant apoprotein and subsequently adsorbed with adult serum to

remove the albumin specificities, King and associates (1975) detected this apoprotein in amniotic fluid throughout gestation, but failed to detect it in human serum, neonatal skin homogenate, nasal mucus, saliva or the first urine excreted by ten infants. A pulmonary origin for this apoprotein in amniotic fluid therefore appears likely. The discovery of a negative correlation between the concentration of apoprotein in amniotic fluid and the subsequent development of respiratory distress syndrome indicates the possibility of using this measurement (or the concentration of one of the constituent proteins) as an index of fetal lung maturity.

It is also possible that some uterine-specific proteins are present in the amniotic fluid. The decidua in particular is a potential source of amniotic fluid proteins due to its close proximity to the amniotic cavity. In addition, evidence from in vitro dialysis experiments indicates that passage of proteins can occur across the "reflected" fetal membranes into the amniotic fluid (Abbas and Tovey, 1960). In this study only the passage of maternal serum proteins across the amnion and chorion was demonstrated. However, there was also evidence that molecular size was the major factor restricting passage of proteins across these membranes. Therefore, it is likely that uterine proteins within the molecular size range for passive diffusion across the fetal membranes will likewise enter the amniotic fluid.

Abnormal levels of uterine proteins present in the amniotic fluid may indicate some uterine dysfunction. However, most uterine disorders associated with pregnancy occur early in gestation and generally prevent implantation of the fertilised ovum or cause abortion soon after implantation. For example, a major uterine dysfunction

during pregnancy is insufficient transformation of the endometrium (Antoine, 1971). This may prevent implantation, or if implantation occurs, the endometrium is unable to support fetal development before the establishment of materno-fetal exchange. Uterine-specific proteins may play an important role in the above mechanism, though this subject is poorly understood as yet.

As previously mentioned, it is possible that some uterine proteins enter the amniotic fluid by passive diffusion. If this is the case, an inverse relationship between their concentrations in amniotic fluid and molecular size would be expected, as is found for maternal serum proteins (Abbas and Tovey, 1960). The presence of uterine proteins in concentrations above the expected levels may indicate active transport of these proteins into the fluid. Such proteins are likely to serve an important function either within the amniotic fluid or within the fetus. Measurement of the levels of uterine-specific proteins in amniotic fluid may therefore provide some indication of fetal well-being. Thus, the search for uterine-specific proteins in amniotic fluid is important because information about the nature and function of such proteins may aid our understanding of maternal-fetal interactions during gestation.

Possible functions of human uterine-specific proteins during gestation

At the present time, the functions of very few human uterine-specific proteins have been established (see section of Introduction entitled "Mammalian uterine proteins."). However, from our limited knowledge of materno-fetal interactions during pregnancy and information

about the uterine proteins of other mammals, several possible functions of human uterine proteins may be outlined.

For example, uterine-specific proteins may be involved in fetal nutrition during the first 3 to 4 weeks of gestation, before vascular exchange is established between mother and fetus. During the pre-implantation period, the developing embryo derives its nutrition from "uterine milk" secreted by the endometrial glands. During implantation, the invading placental trophoblast phagocytoses decidual tissue and blood cells, thus supplying essential proteins to the fetus.

After materno-fetal exchange is established most of the nutritional needs of the fetus are obtained from the maternal circulation via the placenta. It is possible, however, that after this stage some proteins are transferred across the reflected fetal membranes from the parietal decidua into the amniotic fluid and make an additional minor contribution to fetal nutrition.

Other possible functions of human uterine proteins include a role in the regulation of pre- or post-implantation embryonic development. This has been suggested as a possible function for progestagen-dependent endometrial protein (Joshi et al, 1980b), a recently characterised human uterine protein (see section of Introduction entitled "Progestagen-dependent endometrial protein"). There is evidence that uterine-specific proteins serve such a regulatory function in some other mammals. For example, in the cow, four non-serum uterine proteins appear in the uterine fluid just before the phase of rapid embryonic elongation, hence may be involved in the regulation of this process (Roberts and Parker, 1974, 1976; Gibbons et al, 1977). Alternatively,

uterine proteins may be involved in establishing optimal conditions for implantation. There is some evidence that uteroglobin (see section on "Mammalian uterine proteins") may serve a similar function in the rabbit (Krishnan, 1971; Allen and Foote, 1973; McCarthy et al, 1977).

Another important function of human uterine proteins may be the transportation of essential elements from mother to fetus. For example, the purple protein of the pig is believed to be involved in the transport of iron (Bazer, 1975) because each molecule of purple protein binds one atom of iron. The binding and transportation of steroid hormones is an important process during gestation, because the association of oestrogen or progesterone to specific receptor proteins results in de novo synthesis of new proteins which in turn may perform essential functions during pregnancy. Uteroglobin is known to bind progesterone (see section on "Mammalian uterine proteins") and the discovery of a significant correlation between progesterone content and protein content of rabbit uterine fluid during pseudopregnancy suggests that it may serve this function in vivo (Fowler et al, 1976; Borland et al, 1977).

Alpha uterine protein (AUP) : discovery, distribution and properties

Alpha uterine protein is a soluble protein of α_2-mobility on two-dimensional antibody antigen crossed electrophoresis (2D AACE) which is present in both the pregnant and non-pregnant human uterus. AUP was first discovered by Sutcliffe and associates (1978) during a search for novel fetal- or pregnancy-specific proteins in human amniotic fluid. The experimental method used was a subtractive procedure which they

termed "negative antibody affinity chromatography". This involved the passage of pooled amniotic fluid samples of 14-16 weeks' gestation over a series of two Sepharose columns to which were coupled sheep antibodies to human serum proteins, and sheep antibodies to human alphafetoprotein, respectively. The resultant unadsorbed material eluted from the second column contained approximately 3% of the total protein present in the original amniotic fluid pool. This was used to immunise rabbits and an antiserum obtained. The antiserum was found to contain antibodies directed against six non-serum proteins in amniotic fluid, of which one was also specific to human uterine decidua. This protein, designated "alpha uterine protein" was also detected by AACE in endometrium, myometrium, amnion and chorion, but was not detectable in maternal serum or homogenates of human male liver, gut, kidney, lung or skin by this method.

In a subsequent study (Sutcliffe et al, 1980), AUP was purified from 11 week gestation human decidua by a three-step method of affinity chromatography, ion exchange chromatography and gel filtration. Affinity chromatography was performed on a column of Sepharose to which were coupled antibodies raised against amniotic fluid AUP in the previous study. After this step there was a 39% recovery of immunoadsorbed AUP. This material was applied to a column of DEAE-Sepharose and eluted with a linear gradient starting with 500 ml of 10 mM potassium phosphate (pH 7.8) and ending with 500 ml of 10 mM potassium phosphate, 0.2M sodium chloride (pH 6.5). Under these conditions, AUP was eluted from the column at a mean salt concentration of 0.13M NaCl (compared to 0.18M NaCl for serum albumin). From the final gel filtration step on Sephadex G-150, a molecular weight estimate of 50,000 was obtained for AUP.

The resultant purified material was radio-iodinated using ^{125}I and immune precipitates were analysed by SDS-polyacrylamide gel electrophoresis. A single band corresponding to a molecular weight of approximately 23000-25000 daltons was found. These data suggest that AUP is a dimer, composed of two similar or identical polypeptide chains. Evidence of antigenic identity between AUP, decidua and endometrium was obtained by tandem crossed electrophoresis using two different antisera: raised in rabbit against amniotic fluid AUP and decidual AUP, respectively. In addition, AUP from these three sources gave arcs of identical mobility when run separately on two-dimensional AACE plates. Levels of AUP in amniotic fluid do not remain constant throughout gestation. Sutcliffe et al (1978) measured AUP concentration in samples of amniotic fluid between 12 weeks of gestation and term by one-dimensional AACE (see methods section).

A wide range of values was found during this period (see Fig 1). It is evident that in the amniotic fluid samples examined maximal concentrations of AUP occur around 15-18 weeks' gestation, after which there is a gradual decline to term. It must be emphasised, however, that the concentration of AUP in amniotic fluid before 12 weeks of gestation has not been measured, due to the difficulty of obtaining samples of amniotic fluid from early gestation. Therefore, it is possible that the peak concentration of AUP in the fluid may occur during the first trimester, and that the values shown on this graph represent a progressive decline in concentration after the peak.

Possible sites of AUP synthesis, and routes of entry into amniotic fluid

The exact site of synthesis of AUP is not yet known, although

in view of its consistent presence in both pregnant and non-pregnant uterine tissue the uterus appears to be the most likely source. Low concentrations of AUP are also detectable in amnion and chorion, therefore the fetal membranes may be an additional site of synthesis. Alternatively, the AUP present in these membranes may be derived from the decidua.

If AUP is synthesised in uterine decidua there are two possible routes of entry into the amniotic fluid: via the placental barrier or via the reflected fetal membranes. In order to cross the placenta, a decidual protein would have to enter the maternal blood which leaves the spiral arteries in the uterus then circulates between the placental villi and flows along the chorionic plate. It would then have to cross the chorionic plate and associated amnion in order to enter the amniotic fluid. Previous studies have indicated that serum proteins of molecular weight less than 170,000 daltons can permeate into the amniotic fluid (Usategui-Gomez et al, 1966; Gitlin and Biasucci, 1969; Sutcliffe and Brock, 1972) therefore AUP is well within the molecular weight range for passive diffusion across the placental barrier. This route of entry, however, is unlikely for several reasons. Firstly, maternal blood entering the intervillous spaces is under considerable pressure which forces it towards the chorionic plate (Boyd and Hamilton, 1970; Corliss, 1976). Hence the chorionic plate is engulfed with maternal blood which would inhibit free diffusion of uterine proteins across the placental barrier. The inability to detect AUP in maternal serum also argues against this route of entry. Since the chorionic plate and amnion are the only barrier between the maternal circulation and the amniotic fluid this is the most likely route of entry of maternal

serum proteins into the fluid. Therefore, if AUP entered the amniotic fluid along with maternal serum proteins, its concentration throughout gestation would be expected to vary in a similar manner to that of known maternal serum proteins. Clearly, this is not the case for AUP (see following section).

The alternative route of entry of AUP into the amniotic fluid is via the reflected amnion and chorion which lie outwith the placenta in close apposition to the decidua. In this area of the uterus, the reflected membranes are not engulfed by maternal blood because the decidual blood supply does not leave the capillary system. Therefore uterine proteins are more likely to enter the amniotic fluid by this more direct route.

Concentration changes during gestation and possible origins of prolactin α1,4-glucosidase, heat-labile alkaline phosphatase and some serum proteins in amniotic fluid

The concentration profile of AUP in amniotic fluid during the second and third trimesters of gestation is strikingly similar to that of the enzyme α1,4-glucosidase and the pituitary hormone prolactin during this period (Sutcliffe and Brock, 1972; Sutcliffe et al, 1972b; Tyson et al, 1972; Chochinov et al, 1976; Clements et al, 1977). Another enzyme present in amniotic fluid, heat-labile alkaline phosphatase, has an initial peak of activity between 13 and 18 weeks of gestation, followed by a second activity peak towards term. A close correlation has been observed between the levels of α1,4-glucosidase and heat-labile alkaline phosphatase in individual amniotic fluid samples during the early activity peak (Sutcliffe et al, 1972b) which

may indicate a similar tissue of origin for these two enzymes (Sutcliffe and Brock, 1972).

Serum proteins

Alpha uterine protein, prolactin, α1,4-glucosidase and heat-labile alkaline phosphatase are all relatively minor components of amniotic fluid. The variation in concentration throughout pregnancy of these proteins differs markedly from that of the quantitatively major protein components in amniotic fluid, which are of serum origin. This latter group consists of serum albumin and some other relatively low molecular weight serum proteins including α_1-antitrypsin, transferrin and group-specific component (Gc). The concentration profile of these proteins parallels that of the total soluble protein in amniotic fluid, i.e. the concentration rises from about 10 weeks of gestation to reach a maximum between 20 and 30 weeks after which there is a decline to term (Queenan et al, 1970; Sutcliffe and Brock, 1972). Genetic studies indicate that group-specific component, transferrin and orosomucoid are of maternal rather than fetal origin (Ruoslahti et al, 1966; Usategui-Gomez and Morgan, 1966; Seppala et al, 1966; Sutcliffe et al, 1972a; Johnson et al, 1974). In addition, evidence from quantitative studies suggests that most other serum proteins in amniotic fluid are also derived from the mother, and it is widely believed that these proteins enter the amniotic fluid by passive diffusion of maternal serum across the chorionic plate and amnion (Abbas and Tovey, 1960; Strebel, 1960; Derrington and Soothill, 1961; Gitlin et al, 1964; Gitlin and Biasucci, 1969; Sutcliffe and Brock, 1973; Johnson et al, 1974).*

In view of the evidence that serum proteins which reach maximal levels in amniotic fluid between 20 and 30 weeks' gestation have a common maternal origin, it is possible that AUP, prolactin and α1,4-glucosidase which have identical concentration profiles throughout gestation also have a common origin. This may also be the case for heat-labile alkaline phosphatase early in gestation. The exact origin of these four proteins in amniotic fluid, however, is by no means certain.

Prolactin

Several sources of amniotic fluid prolactin have been suggested, including maternal serum (Josimovich et al, 1974; Schenker et al, 1975), fetal serum (Fang and Kim, 1975; Chochinov et al, 1976), placenta (Friesen et al, 1972; McNeilly et al, 1976), placental membranes and decidua (Riddick and Kusmik, 1977; Riddick et al, 1978; Golander et al, 1978; Healy et al, 1979). A serum origin for prolactin is unlikely because the concentration of prolactin in amniotic fluid

* There is some dispute, however, about the origins in amniotic fluid of albumin and α_1-antitrypsin (Brzezinski et al,1964; Kaiser et al, 1974). Brzezinski and associates reported a rare case of fetal bisalbuminemia in which the variant form of albumin was found in the amniotic fluid and fetal serum but was absent from the maternal serum. They concluded that the albumin in amniotic fluid originates from the fetus. There was no evidence, however, that the additional "bisalbumin" peak visualised as a small superimposed peak close to the normal albumin peak was antigenically-related to albumin, and no examination was made of the paternal serum to discover if this trait was inherited. Thus, it is possible that the "bisalbumin" peak was an artefact. A fetal origin has also been proposed for α_1-antitrypsin (Kaiser et al, 1974). This was based on a single case of α_1-antitrypsin deficiency in which a heterozygous mother carrying a fetus homozygous for this trait was found to have amniotic fluid levels of α_1-antitrypsin at term which were less than 10% those of control fluids. Unfortunately, the concentration of α_1 antitrypsin in amniotic fluid was too low for phenotyping to be done.

between 13 and 18 weeks of gestation exceeds that in maternal and fetal sera by about 100-fold (Tyson et al, 1972; Winters et al, 1975; Clements et al, 1977). Towards term, however, the concentration of prolactin in amniotic fluid falls while that in maternal and fetal sera rises, to levels within the same range (Tyson et al, 1972; Winters et al, 1975; Fang and Kim, 1975; Clements et al, 1977), thus a serum origin cannot be discounted at this stage of gestation. Riddick and Kusmik (1977) cultured explants of decidua, placenta and placental membranes obtained from normal pregnancies at delivery and measured the concentration of prolactin in the incubation media and tissue homogenates. In the decidual cultures there was a progressive increase in prolactin concentration in the incubation medium with time, and the total amount of prolactin released during culture exceeded that present in the tissue at the beginning of the incubation period. In contrast, very small amounts of prolactin were released by the other tissues cultured. The authors concluded that de novo synthesis of prolactin by decidua occurred during incubation. In a similar experiment, Golander and associates (1978) found that explants of chorion-decidua secreted prolactin over a 6 day culture period at a relatively constant rate. De novo synthesis was demonstrated directly by the incorporation of radiolabelled amino acids into the prolactin. In addition, cycloheximide treatment resulted in a reduction of the prolactin concentration in the explants and culture media. It therefore appears likely that the decidua and possibly the chorion synthesise and secrete prolactin and that these tissues may be the major source of this hormone in amniotic fluid.

Alpha 1,4-glucosidase and heat-labile alkaline phosphatase

By comparing the concentration gradients of α1,4-glucosidase and heat-labile alkaline phosphatase between serum and amniotic fluid with those of the maternal serum marker Gc, and the fetal serum marker AFP, Sutcliffe and Brock (1972) found that the specific activities of both enzymes were much higher than expected on the basis of either a maternal or fetal serum origin. Other possible sources of amniotic fluid enzymes include maternal non-serum (uterine decidua and myometrium), fetal skin, urine, respiratory and alimentary secretions, amnion, chorion, amniotic fluid cells and umbilical cord. Amniotic fluid cells are unlikely to be the source of α1,4-glucosidase or heat-labile alkaline phosphatase because the cell concentration is low at the time of the early specific activity peak of these enzymes (Nelson and Emery, 1970). In addition, at term when the amniotic fluid cell concentration is maximal (Nelson and Emery, 1970), between 29 and 56 times as much heat-labile alkaline phosphatase is present in the supernatant of amniotic fluid than in the cells (Sutcliffe and Brock, 1972). A fetal origin has been suggested for both these enzymes (Salafsky and Nadler, 1971a,b; Mulivor et al, 1979). Salafsky and Nadler (1971a) detected α1,4-glucosidase activity in the amniotic fluid and kidney of three patients with Pompe's disease, but failed to detect this enzyme in the liver, leucocytes, cultivated fibroblasts or cultivated amniotic fluid cells of these patients. The α1,4-glucosidase present in amniotic fluid and kidney of both control patients and those with Pompe's disease was found to differ from the enzyme present in the other tissues examined with respect to pH optimum and inhibition by turanose. This evidence suggests that the α1,4-glucosidase in amniotic

fluid and kidney is a different enzyme from lysosomal α1,4-glucosidase which is deficient in Pompe's disease. Amniotic fluid α1,4-glucosidase has been further characterised (Fluharty et al, 1973). Other differences between this enzyme and lysosomal α1,4-glucosidase include thermal stability and response to 0.1M potassium chloride. A fetal origin for the α1,4-glucosidase in amniotic fluid is therefore possible. Fetal kidney, however, is unlikely to be a major source of this enzyme because levels in fetal urine are very low between 13 and 18 weeks of gestation (Sutcliffe et al, 1972b). In a further study, Salafsky and Nadler (1971b) reported that 85% of the α1,4-glucosidase activity in second trimester amniotic fluid was localised within intracellular organelles, and suggested that these originate from lysed fetal cells. Other possible sources of amniotic fluid α1,4-glucosidase, for example fetal secretions, uterine decidua and myometrium remain to be investigated.

There are several different types of alkaline phosphatases in human tissues (Suzuki et al, 1969) which can be differentiated according to their thermal stability and inhibition by L-phenylalanine and L-homoarginine. Mulivor et al (1979) investigated the origin of the alkaline phosphatases in amniotic fluid and found that the intestinal type of heat-labile alkaline phosphatase comprises most of the total alkaline phosphatase activity present in amniotic fluid until around 25 weeks of gestation, with a peak of activity occurring around 20 weeks. This coincides with the early activity peak reported by Sutcliffe and Brock (1972). Electrophoretic evidence indicated that this enzyme was of fetal rather than maternal origin. Between

25 and 40 weeks of gestation, however, the greatest proportion of the total enzyme activity is due to the bone, liver, kidney type of heat-labile alkaline phosphatase. A fetal origin is also likely for this form of the enzyme, since there is no corresponding increase of this enzyme in maternal serum at this stage of gestation.

Mammalian uterine proteins

A variety of human uterine-specific proteins have been detected previously (see Aitken, 1979b). Most studies have involved analysis of human uterine fluid throughout the menstrual cycle rather than a direct examination of the proteins present in uterine tissue. In man, and all other mammals examined to date, at the time of ovulation the uterus produces a voluminous secretion which is rich in proteins. (Lutwak-Mann et al, 1960; Fahning, 1965; Aitken, 1970, 1977a; Clemetson et al, 1973; Meglioli et al, 1973). This dilates the uterine lumen thus aiding the movement of sperm towards the oviduct (Aitken, 1979a). In most mammals there is another marked increase in protein content of the uterine secretions during the preimplantation period. These uterine secretions, which comprise a complex mixture of proteins (Surani, 1977; Aitken, 1977b) are thought to be involved in the induction and maintenance of blastocyst activity during implantation (Aitken, 1979a). In some mammals, for example the pig and rabbit, this progestational phase is characterised by the appearance of a dominant non-serum protein in the uterine lumen. In the pig, this dominant component is a purple-coloured glycoprotein of about 32000 molecular weight (Squire et al, 1972; Bazer, 1975) which is synthesised in the cells of the luminal and glandular epithelia of the

endometrium. Maximum synthesis occurs during the midluteal phase, under the influence of progesterone (Chen et al, 1975; Bazer, 1975). The function of this protein is not known: it may have a role in the elongation of the blastocyst or may be involved in the transport of iron (Aitken, 1979b). In the rabbit, the major protein present at this time is a protein variously termed blastokinin (Krishnan and Daniel, 1967) or uteroglobin (Beier, 1968). This protein was first isolated from rabbit uterine fluid, where it reaches maximum concentration on day 5 post-coitum i.e. just before blastulation occurs in vivo (Krishnan and Daniel, 1967). In later studies, immunological identity was established between uteroglobin and "cone-forming" protein, which is present in the oviducal fluid of normal oestrous rabbits (Kay and Feigelson, 1972; Noske and Feigelson, 1976; Feigelson et al, 1977). To date, uteroglobin has been detected in both oestrous and pregnant rabbit oviducal and uterine fluids, endometrium, myometrium, ovary, oviduct, vaginal tissue, blastocoelic fluid and preimplantation blastocyst coverings, male rabbit reproductive tract and flushings, and various non-reproductive sites including lung, trachea, oesophagus, thyroid and bladder (Krishnan and Daniel, 1967; Beier, 1968; Kirchner, 1972, 1976; Hamana and Hafez, 1970; Urzua et al, 1970; Kay and Feigelsen, 1972; Goswami and Feigelson, 1974; Petzoldt, 1974; Beier and Maurer, 1975; Beier et al, 1975; Noske and Feigelson, 1976; Feigelson, 1976; Kirchner and Schroer, 1976; Beato and Beier, 1978; Noske and Gooding, 1978). The biochemical composition of uteroglobin has been determined: it is a globular protein of molecular weight 14000-16000, comprising two similar or identical polypeptide chains, which contains little or no carbohydrate (Murray et al, 1972; McGaughey

and Murray, 1972; Bullock and Connell, 1973; Nieto et al, 1977; Beato and Beier, 1978). The amino acid sequence has been determined (Popp et al, 1978). Oviducal uteroglobin is specifically induced by oestrogens (Kay and Feigelson, 1972; Goswami and Feigelson, 1974; Feigelson, 1976), whereas in the uterus progesterone is the more powerful inducer (Urzua et al, 1970; Bullock and Connell, 1973; Goswami and Feigelson, 1974; Beato and Baier, 1975; Feigelson, 1976). Ovarian steroids do not, however, appear to be involved in the induction of uteroglobin in male reproductive tissues and non-reproductive sites (Feigelson, 1976; Noske and Feigelson, 1976; Feigelson et al, 1977; Daniel and Crowder, 1977). The exact function of uteroglobin is not yet established. It has the ability to bind progesterone and other progestational steroids (Urzua et al, 1970; Rahman et al, 1975; Beato and Baier, 1975; Beato, 1976; Fridlansky and Milgrom, 1976; Beato et al, 1977), hence may be involved in the transport of progesterone. Thus, uteroglobin may function as an intrauterine steroid carrier protein that supplies progesterone to the embryo (Arthur and Chang, 1974) where it could be used as raw material for oestrogen biosynthesis by the trophoblast (Perry et al, 1973). In addition, it has been suggested that uteroglobin may protect the embryo from the effects of free progesterone (Maurer and Beier, 1976) which appears to retard embryonic development when present in physiological concentrations. Uteroglobin itself appears to be embryotoxic if present in the uterus at the wrong stage of development, which suggests that the correct sequence of uterine secretory events is essential for normal embryonic development (Allen and Foote, 1973; Beier, 1976; McCarthy et al, 1977). Although uteroglobin is the quantitatively major protein in the uterine secretions this does not, however, exclude the possibility

that other quantitatively minor proteins may also be important for the establishment of optimal conditions for implantation. There is also evidence that endometrial uteroglobin has proteinase-inhibiting activity, therefore may be involved in the proteolytic system of the uterus (Beier, 1970, 1976; Denker, 1972; Johnson, 1974). Finally, the ubiquitous distribution of uteroglobin suggests that it may have several functions, although these may have some underlying common mechanism (Feigelson, 1976).

In humans there is no corresponding increase in uterine secretory activity after ovulation. In contrast, both the protein concentration and volume of the uterine fluid are higher in the proliferative phase (i.e. pre-ovulation) of the menstrual cycle than in the secretory phase (Maathuis and Aitken, 1978a). The higher protein concentration in the proliferative phase may reflect endometrial secretory activity (Wynn and Harris, 1967). In addition, since oestrogen increases the permeability of the endometrial blood vessels (Pappas and Blanchette, 1965; Martin et al, 1973), direct transudation of protein from the uterine circulation may be a contributary factor. There is evidence that in some mammals the implanting blastocyst may exert a local stimulus on endometrial secretory activity (Daniel, 1972; Renfree, 1972, 1973; Wyatt et al, 1976; Aitken, 1977b). This may also occur in humans, although it awaits investigation.

Several studies have been carried out involving analysis of human uterine fluid throughout the menstrual cycle by polyacrylamide gel electrophoresis (Beier et al, 1970; Shirai et al, 1972; Beier and Beier-Hellwig, 1973; Daniel, 1973; Wolf and Mastroianni, 1975;

Roberts et al, 1976; Maathuis and Aitken, 1978b). Although these reports vary as regards the number of non-serum proteins present and their electrophoretic mobilities, there is general agreement on the absence of a quantitatively dominant non-serum protein at any stage of the menstrual cycle. Maathuis and Aitken (1978b) used an improved flushing technique (Maathuis and Aitken, 1978a) which prevents contamination of uterine fluid samples with blood, cervical mucus, tubal fluid or seminal plasma. They detected a total of 11 non-serum proteins in the uterine flushings, one of which was also present in peritoneal fluid, but absent from plasma. This may indicate some exchange of fluid between the uterine and peritoneal cavities. Similar protein bands were found in uterine fluids from all stages of the menstrual cycle suggesting a lack of an ovarian steroid hormonal control mechanism. In another study of this nature, Roberts and associates (1976) found a total of 6 uterine-specific bands at all stages of the menstrual cycle. One of these bands, a post-transferrin, appeared to increase in intensity during the late secretory phase (days 23-25). Bands of similar mobility have also been reported in uterine fluid (Beier and Beier-Hellwig, 1973; Wolf and Mastroianni, 1975; Maathuis and Aitken, 1978b), and in oviducal fluid (Moghissi, 1970), although Maathuis and Aitken (1978b) did not find any variation in its intensity throughout the cycle, and Wolf and Mastroianni (1975) reported a decrease in the concentration of this protein in post- ovulatory samples.

Several post-albumins are present in human uterine fluid, and it has been suggested that one of these bands is equivalent to uteroglobin (Shirai et al, 1972; Daniel, 1973). In the former study,

a major post-albumin band of similar mobility to rabbit uteroglobin was found in mid-secretory phase samples of human uterine fluid. There was no evidence however, of immunological or biochemical identity between this protein and uteroglobin, and the technique used to collect the uterine fluid samples failed to exclude blood contamination (Beier and Beier-Hellwig, 1973; Roberts et al, 1976; Maathuis and Aitken, 1978b). This is an important factor because the electrophoretic mobility of uteroglobin is similar to that of haemoglobin (Roberts et al, 1976). Daniel (1973) also detected a band of identical mobility to purified rabbit uteroglobin in a mid-secretory phase sample of human uterine fluid and presented evidence of immunological identity between this uterine flushing and blastokinin using a virtually monospecific antiserum raised against rabbit uteroglobin. He failed to give any evidence however, that the band visualised on the gel and the protein which cross-reacted with uteroglobin were in any way related.

In a more recent study, Noske and Feigelson (1976) claimed to have detected a cross-reaching antigen to "cone-forming" protein in human endometrium, uterine and oviducal flushings and seminal plasma. This was based on evidence from double immunodiffusion, radial immunodiffusion and immunoelectrophoresis experiments. The double immunodiffusion experiments were particularly interesting, in that a single line of identity was found between a female rabbit reproductive tract preparation and each of human progestational phase endometrial extract and seminal plasma using monospecific goat antiserum to rabbit cone-forming protein (CP). No evidence was found of antigenic identity between rabbit CP and human oviducal or uterine flushings using this method. On immunoelectrophoresis, however, human oviducal and uterine

flushings and progestational phase endometrial extracts gave precipitin arcs of similar mobilities to rabbit CP, when reacted against anti-rabbit CP serum. A "shoulder" was observed on the precipitin arcs formed by the human material, but was absent from the rabbit CP arcs. The authors suggested that this was indicative of electrophoretic variants of CP in the human material. However, in view of the lack of evidence of cross-reactivity between rabbit CP and human oviducal and uterine flushings, it is difficult to compare the human protein(s) visualised on immunoelectrophoresis with rabbit CP. These observations, although interesting, are as yet unconfirmed.

A variety of proteins have been detected in human uterine tissue, notably oestrogen-induced protein and two steroid-hormone receptor proteins. Villaneuva and Heinrichs (1977) reported the appearance of a protein in human endometrium following oestrogen administration. Induction of this protein occurred in both secretory and proliferative phase endometrium. Treatment with actinomycin-D prevented the appearance of this protein, suggesting that RNA-synthesis is an essential step in the induction process, i.e. de novo synthesis occurs following oestrogen administration. This protein appears to be very similar to an oestrogen-induced protein (IP) present in rat uterus (Notides and Gorski, 1966). Induced protein, which is also a product of de novo synthesis in the uterus (De Angelo and Gorski, 1970; Katzenellenbogen and Gorski, 1972), has been isolated and a preliminary characterisation performed. This protein consists of a single homogeneous polypeptide chain of molecular weight 45000, and is acidic in nature (Iacobelli et al, 1973). The physiological function of IP is unknown. It has been suggested, however, that IP has a role in the

mechanism of gene expression, possibly by facilitating further RNA synthesis or transport, which would then lead to the final expression of steroid action (Baulieu et al, 1972; Du Pont-Mairesse and Galand, 1975).

Both oestrogen- and progesterone-receptors are present in the mammalian uterus and the information available on these proteins has recently been reviewed (Janne et al, 1978). These receptors are found in both myometrium and endometrium of the human uterus and their physicochemical characteristics and ligand-binding specificities have been established (Jensen et al, 1969; Puca et al, 1971, 1972; Martin, 1972; Notides et al, 1972; Verma and Laumas, 1973; Ratajczak and Hahnel, 1974; Young and Cleary, 1974; Janne et al, 1975; Smith et al, 1975; Sanborn et al, 1975). Levels of both receptors in the mammalian uterus are under hormonal control. Oestrogens stimulate the formation of both oestrogen- and progesterone-receptors, whereas progesterone and progestins decrease the concentration of both receptors (Sarff and Gorski, 1971; Milgrom et al, 1972, 1973; Anderson et al, 1973; Leung and Sasaki, 1973; Mester et al, 1974; Freifeld et al, 1974; Mester and Baulieu, 1975; Hsueh et al, 1975; Luu Thi et al, 1975; Clark et al, 1977; Vu Hai et al, 1977; Leavitt et al, 1974, 1977). In addition, in the human uterus concentrations of both steroid-hormone receptors are higher during the proliferative phase of the menstrual cycle than during the secretory phase (Haukkamaa and Luukainen, 1974; Bayard et al, 1975; Janne et al, 1976; Tseng et al, 1977; Syrjala et al, 1978).

Progestagen-dependent endometrial protein

There have been relatively few studies on human uterine proteins synthesised during gestation. Recently, however, Joshi and associates reported the discovery of a glycoprotein in human secretory phase endometrium and early gestation decidua (Joshi et al, 1980a,b), using an immunological method of detection. They failed to detect this protein in proliferative phase endometrium, term placenta or adult serum by this method. Using a more sensitive double-isotopic labelling technique, however, synthesis of this protein was demonstrated in early gestation decidua and endometrium from both phases of the menstrual cycle, although the rate of synthesis was much lower in proliferative phase endometrium than in the other two tissues. The authors therefore suggested that synthesis of this glycoprotein may be regulated mainly by progesterone. Thus, the presence of progesterone in the serum throughout the menstrual cycle (Moghissi et al, 1972) may account for the small amounts of this protein found in proliferative phase endometrium. Due to its consistent presence in uterine tissue during the progestational phase, the authors named this protein "progestagen-dependent endometrial protein" (PEP).

Following its initial discovery, PEP was partially purified from the cytosol of decidua-rich tissues by Con-A Sepharose chromatography and polyacrylamide gel electrophoresis, then further characterised (Joshi et al, 1980b). Immunological reactivity of PEP was not affected by exposure to temperatures of 40-85^{o}C or values of pH from 2-12. Treatment with the enzymes pepsin, DNase, RNase and neuraminidase had no effect, whereas trypsin completely destroyed antigenicity, indicating that the antigenic determinants are located within the protein molecule

rather than in the terminal residues. Although pepsin is also an endopeptidase, it attacks the peptide chain at different points from trypsin and is less specific. Bonds with aromatic or acidic amino acids are cleaved preferentially, and in addition neighbouring amino acids appear to influence pepsin activity. Trypsin specifically cleaves lysyl or arginyl bonds such that the resulting peptides have either lysine or arginine as the carboxy-terminal amino acid. This may indicate that one or both of these bonds contributes to the antigenic site(s) of PEP. The molecular weight of PEP was estimated by gel filtration to be approximately 48000 daltons. Dissociation with sodium dodecyl sulphate under reducing conditions resulted in a single immunoreactive component of about 27000 molecular weight. It is therefore possible that PEP is a dimer. No evidence was found of immunological identity between PEP and any of the following proteins: transferrin, ceruloplasmin, α_1-antitrypsin, ferritin, uteroglobin, alpha fetoprotein, human chorionic gonadotrophin, pregnancy-associated plasma proteins A and C, or pregnancy-zone protein. In addition, antiserum raised in rabbit against PEP failed to react against the decidual cytosols of pregnant baboons or pseudo-pregnant rats.

Progestagen-dependent endometrial protein is similar to alpha uterine protein in several respects. Firstly, both proteins are thought to be uterine-specific, although the complete tissue distribution is not yet known for either protein. In addition, PEP and AUP are similar as regards molecular size and subunit structure. It therefore appears likely that AUP and PEP are the same protein, and investigations into this possibility are reported in this thesis.

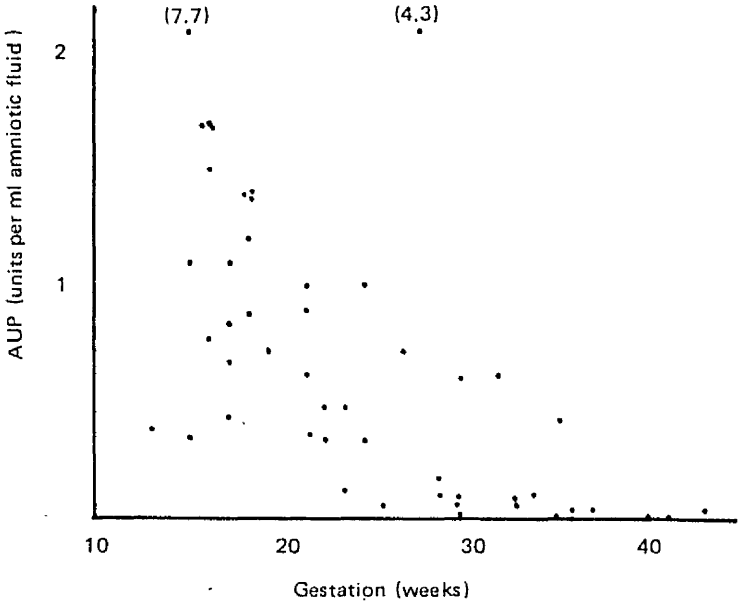


Fig. 1 (Adapted from Sutcliffe et al, 1978). The concentration of AUP in separate samples of human amniotic fluid obtained from normal pregnancies during the 2nd and 3rd trimesters. AUP was measured in samples of amniotic fluid (2-10 µl) by one-dimensional AACE. The area under each precipitin peak was measured and the concentration of AUP expressed in units of area (mm^2) per ml amniotic fluid. Internal standards were run simultaneously on each AACE plate to control for the reproducibility of the assay.

Aims of project

The overall purpose of this research was to further study and characterise the protein known as alpha uterine protein (AUP). The individual aims pursued during this project were as follows:

1) To compare by biochemical and immunological means AUP from the two major sources known, namely uterine decidua and amniotic fluid.

2) To establish more fully the tissue distribution of AUP.

3) To determine whether levels of AUP in uterine decidua vary during gestation in a similar manner to levels in amniotic fluid.

4) To purify AUP from amniotic fluid since this is a more-readily available source of AUP than uterine decidua.

5) To investigate the source of AUP in amniotic fluid.

6) To determine whether AUP is the same protein as that known as "progestagen-dependent endometrial protein" (PEP) (Joshi *et al*, 1980a,b).

7) To attempt to establish the biological function of AUP.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, ovalbumin, β-lactoglobulin, myoglobin, carbonic anhydrase, Blue dextran, hydrocortisone (cortisol), Coomassie Brilliant Blue R stain, agarose (Type 1. Low electro-endosmosis), 2-mercaptoethanol and Trizma base (Tris) were obtained from the Sigma Chemical Company. In the preparation of gels and buffers chemicals of analar grade were used. Most of these chemicals were obtained from either BDH Chemicals Ltd., or Hopkins and Williams.

Antisera

Monospecific rabbit antisera to Human Albumin, Gc-globulin and α_1-antitrypsin were obtained from Behringwerke AG, Marburg, W. Germany. Sheep antiserum to whole human serum proteins was previously raised in this laboratory. Rabbit antisera to AUP from two separate sources, amniotic fluid and uterine decidua were available in the laboratory. The antiserum to amniotic fluid AUP was produced in this laboratory. Rabbits were injected with unadsorbed protein from human amniotic fluid which had been subjected to "negative antibody affinity chromatography" in order to remove the major serum proteins and alpha fetoprotein, as described by Sutcliffe *et al* (1978). This antiserum was rendered monospecific to AUP by adsorption with adult human serum and adult liver homogenate. The antiserum to decidual AUP was produced by the Radiochemical Centre, Amersham. This antiserum was raised against AUP partially-purified in this laboratory from 11 week gestation human decidua. The purification procedure consisted of antibody affinity chromatography followed by ion exchange chromatography, as described by Sutcliffe *et al* (1980). However, the final gel

filtration step was omitted. Instead, AUP was isolated on non-denaturing polyacrylamide tube gels and subsequently eluted from the gels. This material was used to immunise a total of three rabbits.

Adsorption of anti-AUP serum

Unless otherwise stated, anti-AUP serum was routinely adsorbed with adult human serum before use, in order to remove serum specificities. Adsorption was performed in the solid phase using a glutaraldehyde polymer immunoadsorbent, according to the method of Avrameas and Ternynck (1969). The immunoadsorbent was prepared as follows: 25% glutaraldehyde was added to undialysed adult human serum, in a ratio of 1 to 50 (v/v). The serum was placed in a small beaker and the glutaraldehyde added dropwise, with stirring. An insoluble gel formed within 5-10 minutes. This was left overnight at room temperature then homogenised in phosphate-buffered saline (PBS) in an MSE blender for several minutes. This was followed by centrifugation at 5K for 10 minutes in a refrigerated centrifuge (Sorvall RC-2, Du-Pont Instruments). The protein concentration in the supernatant was measured spectrophotometrically (optical density at 280 nm) to test the degree of protein insolubilization. (Under these conditions, most of the protein should be insolubilized, hence there should be a low protein concentration in the supernatant.) The supernatant was then discarded. The insoluble pellet was pressed through a metal sieve into a large beaker and dispersed in PBS. The resultant polymer was stirred on a mechanical mixer until it resembled "fines". It was then allowed to settle and the supernatant decanted off. The polymer was then stored at $4^{o}C$ until use. Term cord serum-glutaraldehyde

polymer was produced in the same way, using a 50:1 ratio of undialysed term cord serum to 25% glutaraldehyde instead of adult human serum.

Use of glutaraldehyde polymer immunoadsorbents: A volume of polymer approximately twice that of the antiserum to be adsorbed was placed in a test tube and packed by high speed centrifugation for about 2 minutes using a bench centrifuge. The supernatant was discarded and the antiserum pipetted on to the polymer. The contents of the test tube were thoroughly mixed using a Whirlimixer then agitated overnight at room temperature on an orbital shaker to allow adsorption to occur. The polymer was subsequently separated from the adsorbed antiserum by centrifugation.

Homogenisation of Tissues

Homogenates of decidual and placental tissue were prepared. The excised pieces of tissue were rinsed with PBS to remove blood clots and extraneous proteins then blotted dry. All tissue samples were homogenised in an equivalent volume of PBS. Small pieces of tissues were homogenised by hand in a glass vessel with a ground glass plunger. Larger tissue samples were homogenised in an MSE blender. The resultant homogenates were centrifuged at 13K for 15 minutes in the Sorvall refrigerated centrifuge. The supernatant was then filtered through Whatman No. 1 paper to remove any surface lipids. A clear homogenate was thus obtained.

Estimation of protein concentration

This was performed according to the spectrophotometric method described by Lowry et al (1951). A calibration graph was first

constructed using increasing volumes (20, 50, 80 and 100 µl) of bovine serum albumin, of protein concentration 1 mg/ml as the reference standard. The protein concentration in each sample assayed was then calculated as the mean of three determinations performed using different sample volumes.

Buffers

10 mM phosphate buffers: These were prepared by adding 10 ml of solutions of 1 M potassium dihydrogen orthophosphate (KH_2PO_4) and 1 M di-potassium hydrogen orthophosphate tri-hydrate (K_2HPO_4, $3H_2O$) per litre of distilled water. The ratio of the volumes of 1 M KH_2PO_4 and 1 M $K_2HPO_4, 3H_2O$ varied according to the pH of the buffer required. 10 mM phosphate buffers containing sodium chloride at 50 mM-0.5 M concentrations were prepared in the same way. In these buffers adjustments of pH were made by the addition of HCl or NaOH, as required.

2M potassium iodide, 50 mM Tris, pH 8.0 (for antibody affinity chromatography).

332 g KI
6.05 g Tris
} In 1 litre of phosphate buffered saline
To pH 8.0 with HCl

Page acetate buffer (for Con-A Sepharose chromatography)

0.1 M	sodium acetate	16.406 g
1.0 M	sodium chloride	116.88 g
1.0 mM	calcium chloride	0.22198 g
1.0 mM	manganous chloride	0.39582 g
1.0 mM	magnesium chloride	0.40666 g

In 2 litres of distilled water. To pH 6.0 with HCl.

Barbitone immunoelectrophoresis buffers

	1 x conc. Tank buffer	2 x conc. Gel buffer
Barbitone	13.8 g	11.07 g
Sodium barbitone	87.6 g	70.07 g
Calcium lactate	3.84 g	10.24 g
Merthiolate (thiomersal)	2.0 g	1/5000 *
Tris	121.1 g	-
Volume	10 L	5 L
pH	8.6	8.6

* Use 1 L. of 1:1000 merthiolate per 5 L. gel buffer.

Immunological Techniques

Antibody-antigen crossed electrophoresis (AACE) was carried out on 1% agarose gel according to the methods of Laurell (1965, 1966) and Clarke and Freeman (1968). Ouchterlony double immunodiffusion was also performed on 1% agarose gel, as described by Ouchterlony and Nilsson (1978).

Preparation of 1% agarose gel plates: Agarose was prepared by autoclaving 1 g agarose with 50 ml distilled water. While the agarose was still molten, 50 ml of 2 x concentrated gel buffer was added and thoroughly mixed. During use, bottles of agarose were placed in a water bath at 50-60^{o}C to retain the molten state. Both AACE and double immunodiffusion were carried out on glass plates approximately 8 cm square which hold 10 ml gel. Molten agarose was pipetted on to the plates to form an even layer, and allowed to solidify. The best results were obtained if the poured plates were left in a humidity cabinet for several hours before use, to allow the gel to set completely.

One-dimensional AACE: A horizontal strip of gel was removed from the gel plate and replaced by a mixture of molten agarose and antiserum to form the antibody bed. Antiserum concentrations of up to 20% by volume may be added to the molten gel. A series of wells were then cut in the gel under the antibody bed and small volumes (generally 2-10 µl) of the antigens introduced into each well using a Hamilton microlitre syringe. Electrophoresis was then carried out in a perspex tank containing Barbitone tank buffer, pH 8.6, such that the antigens migrated across the antibody bed towards the anode. Electrophoresis was performed overnight at 4°C at a constant current of 3 mA/plate.

Two-dimensional AACE: In this technique, proteins are separated according to charge in the first dimension then electrophoresed into an antibody bed. Therefore it may be used to separate the components in a complex mixture of antigens or to establish the electrophoretic mobility of a single antigen. The antigen was introduced into a well cut at one side of the gel and electrophoresed in the anodal direction at a constant voltage of approximately 20 mV/cm to form the first dimension. Electrophoresis in the first dimension generally took about 3 hours. The migration of the antigen in question was monitored relative to that of albumin which has a greater mobility than that of most serum proteins. The addition of tincture of merthiolate, which contains an orange dye to the gel enables albumin to be easily located. Albumin binds to this dye and is visualised as a pink spot. This was marked by a small circle cut into the gel using the tip of a Pasteur pipette. After the first dimension electrophoresis was completed, an antibody bed was poured parallel to the protein track. The plate was then rotated 90° such that the antigen(s) was electrophoresed into

the antibody bed. This second dimension was run more slowly at a constant current of 3 mA/plate, overnight.

Ouchterlony double immunodiffusion: This was performed according to the method of Ouchterlony and Nilsson (1978). Circular wells of 4 mm diameter were cut in 1% agarose plates and solutions of antisera and antigens introduced into separate wells. The wells were generally spaced about 8 mm apart (centre to centre), but the spacing varied according to the concentrations of the reagents used. The plates were left in a humidity cabinet for 24-48 hours to allow immunodiffusion to occur. Lines of precipitation mark the formation of immune complexes. These were sometimes visualised without staining as white lines on the gel.

Both AACE and Ouchterlony plates were washed individually in 10% PBS for approximately 24 hours, with several changes. During washing the buffer was circulated using an air pump. This caused the gel sheet to detach from the glass plate thus enabling washing of both sides of the gel. After washing, each gel was carefully replaced on its glass plate and a sheet of blotting paper placed on top. The plates were then dried in air. The blotting paper prevented shrinkage of the gel from the edges of the glass plate. The gels were then stained with 2% Coomassie Brilliant Blue. This stain consists of 2% Coomassie Brilliant Blue R Stain (w/v), 50% Methanol (v/v) and 7% acetic acid (v/v). Finally, the gels were destained in 50% Methanol (v/v), 7% acetic acid (v/v). Immune-complexes were visualised as blue-coloured precipitin arcs.

Quantitation of proteins by one-dimensional AACE

The concentration of a given protein may be measured by 1 D

AACE using an antiserum specific to this protein. In this technique, various volumes of the antigen in question are applied to a 1 D AACE plate and electrophoresis performed as described in the preceding section. A reference standard containing a known concentration of the protein to be measured is run alongside the unknown sample (preferably on the same AACE plate). The areas under the resultant precipitin peaks are then measured. From a graphical expression of volume applied (μl) versus area enclosed by the peaks (mm^2), the concentration of the protein in the unknown sample can be calculated relative to that in the reference standard.

Worked example: The concentration of α_1-antitrypsin in a sample of human amniotic fluid was measured relative to that in human serum (Protein-Standard-Serum LC-V, Behringwerke AG, Marburg, W. Germany). The latter was used as a reference standard because it is known to contain 0.13 mg/ml α_1-antitrypsin. Fig 2(a) shows the 1 D AACE quantitation plate: the five wells on the right hand side of the plate contain different volumes of the serum standard and the five wells on the left hand side corresponding volumes of amniotic fluid. The areas under the precipitin peaks are tabulated in Fig 2. These results are expressed graphically in Fig 2(b). The concentration of α_1-AT in amniotic fluid can be directly compared to that in human serum by comparing the slopes of the lines on the graph. This gives a value of 2.32. Hence, the concentration of α_1-AT in the amniotic fluid sample is 2.32 times that in the standard serum sample. Therefore the concentration of α_1-AT in the amniotic fluid sample is 2.32 x 0.13 = 0.30 mg/ml. Note that in the case of AUP quantitations by 1 D AACE no reference standard of known AUP concentration was available. Therefore levels of AUP in unknown samples

were compared with those in an arbitrary standard which was said to contain 1 "unit" of AUP per ml. The results were thus expressed as "units" AUP/ml or "units" AUP/mg protein.

SDS-polyacrylamide gel electrophoresis

4.5-12.5% gradient SDS-polyacrylamide slab gel electrophoresis was kindly carried out by Mr. J. Hunter according to the method of Laemmli (1970). Details of the reagents, buffers and gel composition are outlined in Table 1. In pouring the slab gel the bottom of the gel was sealed with a layer of 12.5% running gel to a depth of approximately 1.5 cm. This was allowed to set, then the 4.5-12.5% gradient running gel poured. The depth of this layer was approximately 13 cm. This was allowed to set as before, then the final layer of 4.5% spacer gel poured to a depth of about 2.5 cm. The loading comb was inserted before the gel set then removed when setting had occurred. Preparation of the samples run on the gel is described in the "Results" section. The gel was run at 30-35 μA without cooling for approximately 5 hours, followed by 4 hours at 40-45 μA, during which time the gel was air-cooled using an electric fan.

Binding Theory and Scatchard Plot

Scatchard analysis was carried out as described by Rosenthal (1967). This is outlined below:

Binding Theory

(b) = concentration of ligand bound to macromolecule

(u) = concentration of free ligand

(M) = concentration of macromolecule

n = number of binding sites (this can only be calculated if using a protein preparation of known molecular weight)

k = intrinsic association constant

(1) $k = \frac{(b)}{(u) \times \text{conc. of free binding sites}}$

(2) or, $\frac{(b)}{(u)} = k \times \text{conc. of free binding sites}$

(3) Conc. of free binding sites ≡ conc. of available binding sites - conc. of occupied binding sites.

(4) Therefore, $\frac{(b)}{(u)} = k\,(n.\,(M) - (b))$

Scatchard plot. Equation (4) is obtained by plotting $\frac{(b)}{(u)}$ against (b), which gives a straight line with a slope of -k, an intercept on the ordinate of k.n.(M) and an intercept on the abscissa of n.(M), where n.(M) ≡ conc. of binding sites. Therefore, if (M) is known, n can be calculated. The Scatchard Plot obtained from this equation is illustrated in Fig. 3.

NB/ If a curved line is obtained on the graph, this represents the sum of two classes of binding: specific (initial steep descent of curve ie high affinity binding) and non-specific (low affinity binding). This may reflect the presence of a single macromolecule with more than one group of binding sites or two or more macromolecules with differing binding affinities.

Fig 2. Quantitation of α_1-antitrypsin in human amniotic fluid by one-dimensional AACE.

a) One-dimensional AACE quantitation plate comparing the concentration of α_1-antitrypsin in human amniotic fluid and standard human serum. The antiserum was monospecific rabbit antiserum to human α_1-antitrypsin, used at a final concentration of 4%. The antigens were as follows:

Wells 1-5 : Human amniotic fluid (2,4,6,8,10 µl)

Wells 6-10 : Standard human serum (2,4,6,8,10 µl).

Areas under the precipitin peaks

Volume applied (µl)	Area under peak (mm^2)	
	Amniotic fluid	Standard serum
2	12	3
4	19	7
6	28	10
8	34	17
10	45	20

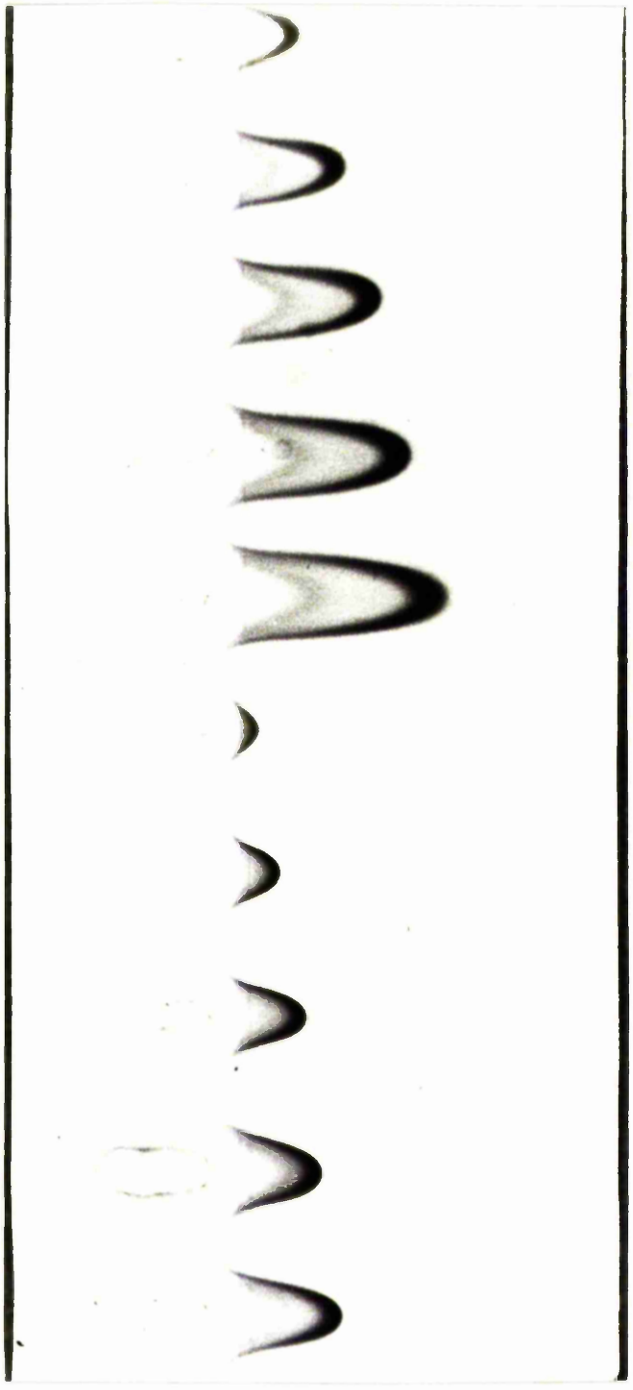


Fig. 2a)

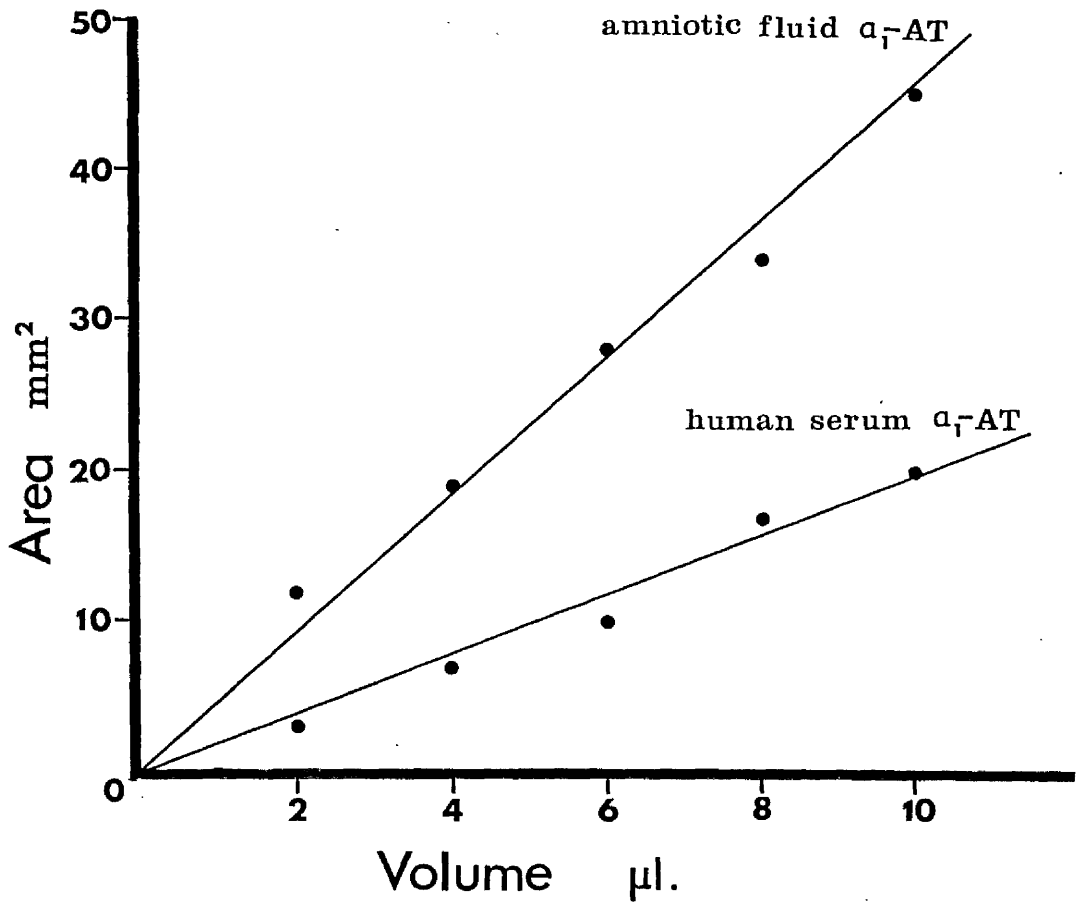


Fig. 2(b) Graph of areas under precipitin peaks against volume applied on AACE.

Table 1. Reagents, buffers and gels used in 4.5-12.5% gradient SDS-polyacrylamide gel electrophoresis.

Reagents:

30 g acrylamide, 0.8 g Bis acrylamide — to 100 ml with distilled water

10% ammonium persulphate (freshly-prepared)

Temed

Glycerol : water (1 + 1 solution)

Buffers:

Running gel buffer : 18.15 g Tris, 0.4 g SDS — to 100 ml with distilled water. Adjusted to pH 8.9 with HCl.

Spacer gel buffer : 5.9 g Tris, 0.4 g SDS — to 100 ml with distilled water. Adjusted to pH 6.7 with HCl.

Tank buffer : 6.3 g Tris, 4.0 g glycine, 1.0 g SDS — to 1 L. with distilled water

4.5-12.5% gradient gel

Reagent	4.5% gel	12.5% gel
Acrylamide	3.6 ml	10 ml
Running or Spacer gel buffer	6.0 ml	6.0 ml
Glycerol (1 + 1 soln)	-	7.2 ml
Distilled H_2O	14.4 ml	0.6 ml
Temed	20 µl	20 µl
10% Ammonium persulphate	0.1 ml	0.1 ml
Total volume approx	24 ml	24 ml

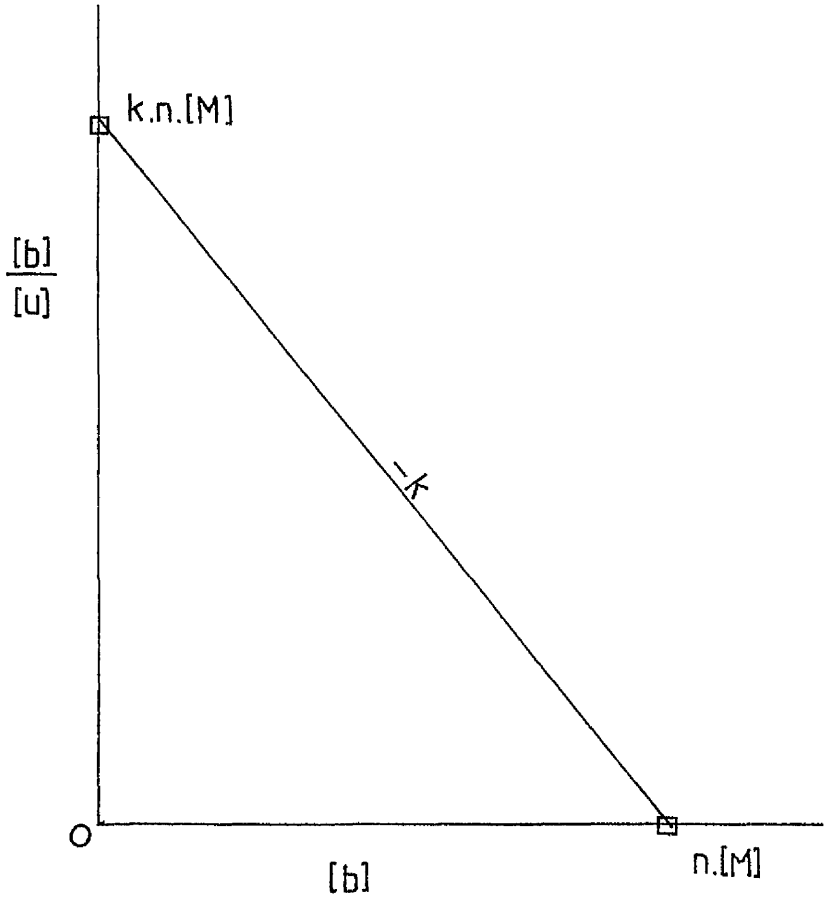


Fig. 3. Scatchard plot.

RESULTS

1. Comparison of AUP from uterine decidua and amniotic fluid

Introduction

Although AUP was first isolated from amniotic fluid (Sutcliffe et al, 1978), it was subsequently purified from uterine decidua and the initial physicochemical characterisation was based on this decidual AUP (Sutcliffe et al, 1980). Evidence was also presented of immunological and electrophoretic identity between AUP from these two major sources (Sutcliffe et al, 1980). However, to date this evidence remains unconfirmed. Therefore, in an attempt to substantiate these findings, AUP from uterine decidua and amniotic fluid was compared as regards molecular size, electrophoretic mobility and immunological cross-reactivity.

a) Determination of the molecular weight of amniotic fluid AUP

Methods: A 3.5 x 90 cm column of Sephadex G-150 (Pharmacia) was calibrated by applying the following pure proteins of known molecular weight as reference standards: bovine serum albumin, ovalbumin, β-lactoglobulin and myoglobin. Fractions were collected after each run of the column and the elution volumes (Ve) estimated by spectrophotometry (absorbance at OD280). The void volume (Vo) was estimated by applying Blue dextran to the column. This has a molecular weight of 2×10^6 which is above the exclusion limit of the gel. A calibration curve was then drawn from a graph of (Ve-Vo) versus log MW. A 50 ml sample of amniotic fluid (pooled fluids of 14-16 weeks' gestation) was dialysed against 10% PBS then lyophilised in an Edwards Modulyo Freeze-drier and resuspended in 5 ml distilled water. The final protein concentration in the amniotic fluid sample was 11.5 mg/ml. After centrifugation, the supernatant was applied to the column and fractions collected

as before. The presence of AUP in the fractions was detected by one-dimensional AACE using two different antisera: raised in rabbit against amniotic fluid AUP and decidual AUP, respectively. The elution volume of AUP was estimated from the fractions which produced the highest precipitin peaks on 1D AACE.
(see main "Materials and Methods" section for further details.)

Results: The molecular weights and elution volumes of the proteins used to establish the calibration curve and the elution volume of AUP are shown in Table 2. The elution curve of myoglobin, one of the reference standards, is illustrated in Fig 4. The amniotic fluid fractions which contained AUP were detected by one-dimensional AACE. The same peak fractions were observed using antisera raised against either decidual or amniotic fluid AUP. Fig 5 shows the peak AUP fractions as visualised on one-dimensional AACE. An elution volume of 303.3 ml was measured for AUP. When the Ve-Vo (AUP) was plotted on the calibration curve, it was found to correspond to a molecular weight of 53,700 (see Fig 6).

b) Comparison of decidual and amniotic fluid AUP as regards electrophoretic mobility and immunological cross-reactivity

Methods: The electrophoretic mobilities of AUP from these two sources were examined by two-dimensional AACE. Immunological cross-reactivity was tested using two different methods: Ouchterlony double immunodiffusion and tandem-crossed electrophoresis. In the latter technique, the two antigens to be tested for cross-reactivity, in this case amniotic fluid and decidua are run simultaneously on two-dimensional AACE. The two antigens are spaced about 8 mm apart horizontally and electrophoresed

in the first dimension. If the antigens are identical they will have the same electrophoretic mobility, but due to the horizontal spacing one will "lag behind" the other. The plate is then turned 90^{o} and the second dimension electrophoresis carried out as described previously. If the two antigens cross-react completely the resultant precipitin peaks will be continuous. However, two overlapping peaks will indicate either partial identity or non-identity between the antigens, depending on the degree of overlap. (see main "Materials and Methods" section for further details.)

Results: AUP from the two sources examined gave single peaks of identical mobility (see Fig 7). Peak morphology was similar in each case, in that a somewhat "skew" shape was observed. On Ouchterlony double immunodiffusion a reaction of complete identity was observed between the antisera raised against AUP from uterine decidua and amniotic fluid (see Fig 8). In addition, a precipitin peak with a continuous "shoulder" was observed on tandem-crossed electrophoresis, indicating complete identity between AUP from these two sources (see Fig 9).

Discussion: AUP from uterine decidua and amniotic fluid was found to have the same electrophoretic mobility on two-dimensional AACE and to be immunologically identical. This supports the previous findings of Sutcliffe et al (1980). In addition, the molecular weight of AUP of amniotic fluid origin was estimated by gel filtration on Sephadex G-150 (Pharmacia) to be approximately 53,700 daltons. This compares favourably with the MW of 50,000 previously found for decidual AUP by the same method (Sutcliffe et al, 1980). Thus, it appears likely that AUP from these two major sources is antigenically and structurally

identical. However, unequivocal evidence of identity between AUP from amniotic fluid and decidua awaits a detailed comparison of the physico-chemical characteristics of the pure protein from each source. This cannot be carried out at this stage because to date AUP has only been purified from human uterine decidua (Sutcliffe et al, 1980). However, efforts will be made during the course of this research project to purify AUP from human amniotic fluid. (see Results 6. "The partial purification of AUP from amniotic fluid.")

Table 2. The elution volumes of molecular weight standards and AUP after gel filtration on Sephadex G-150

Standard	Molecular weight	Elution vol (ml) (Ve)	Elution vol - Void vol (ml) (Ve-Vo)
Blue dextran	2×10^6	201.8	0
BSA	68000	282	80.2
Ovalbumin	43000	308.5	106.7
β-Lactoglobulin	36726	332	130.2
Myoglobin	16951	369.5	167.7
AUP	-	303.3	101.5

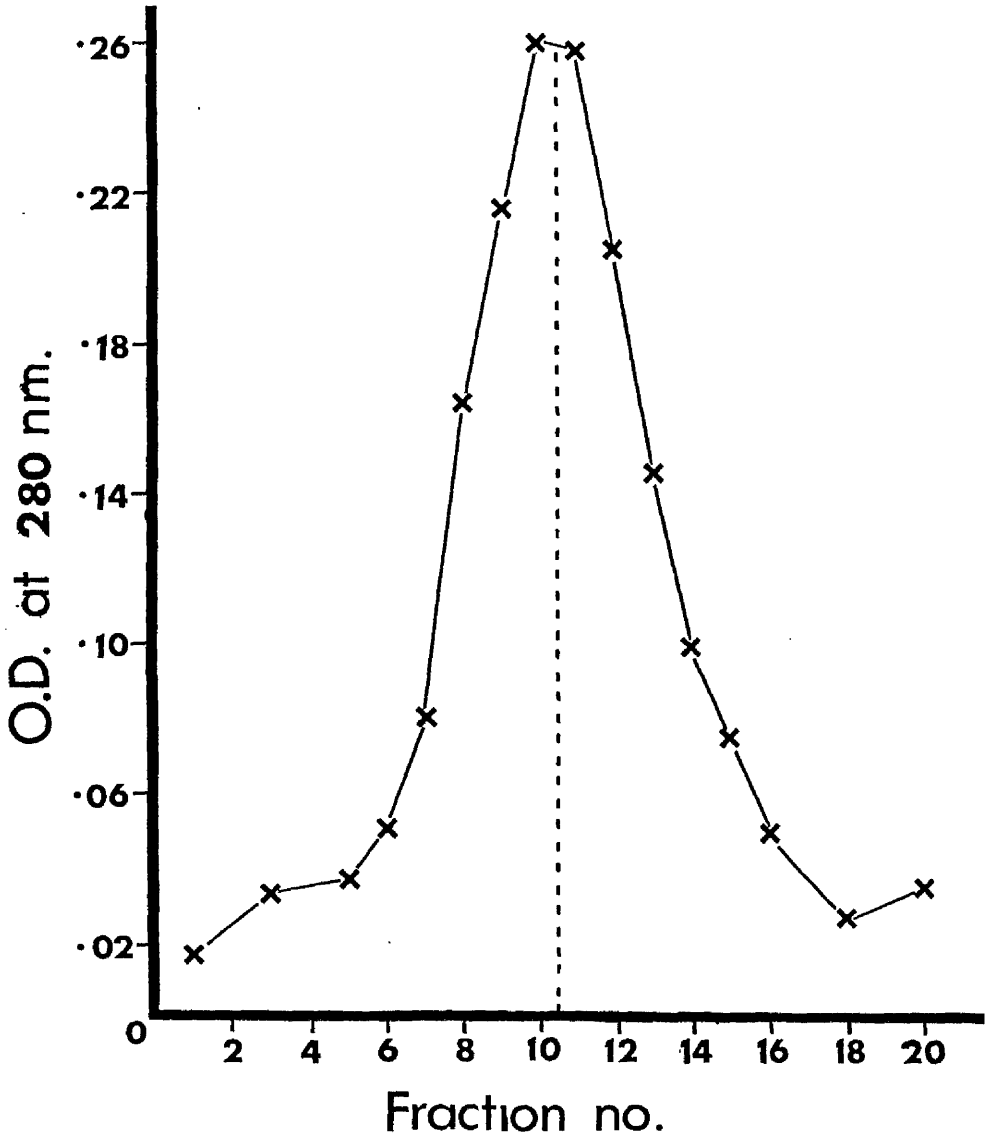


Fig. 4. Elution curve of Myoglobin after gel filtration on Sephadex G-150. The elution volume (Ve) is indicated by a broken line.

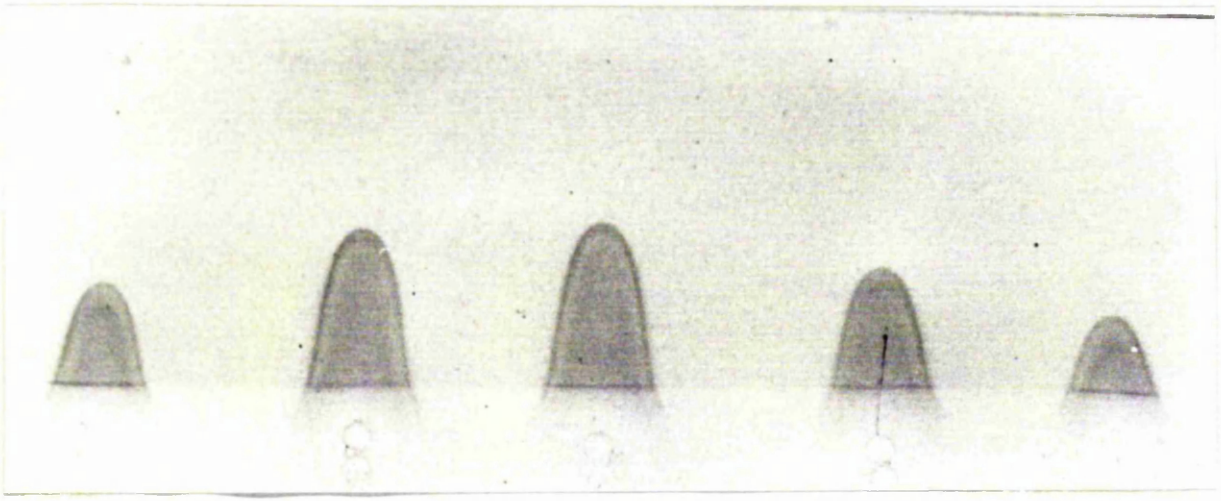


Fig. 5. One-dimensional AACE of peak AUP fractions eluted after gel filtration of amniotic fluid on Sephadex G-150. The antiserum was raised against decidual AUP and adsorbed with adult male human serum. It was used at a final concentration of 10%. 10 µl volumes of each fraction were applied to separate antigen wells.

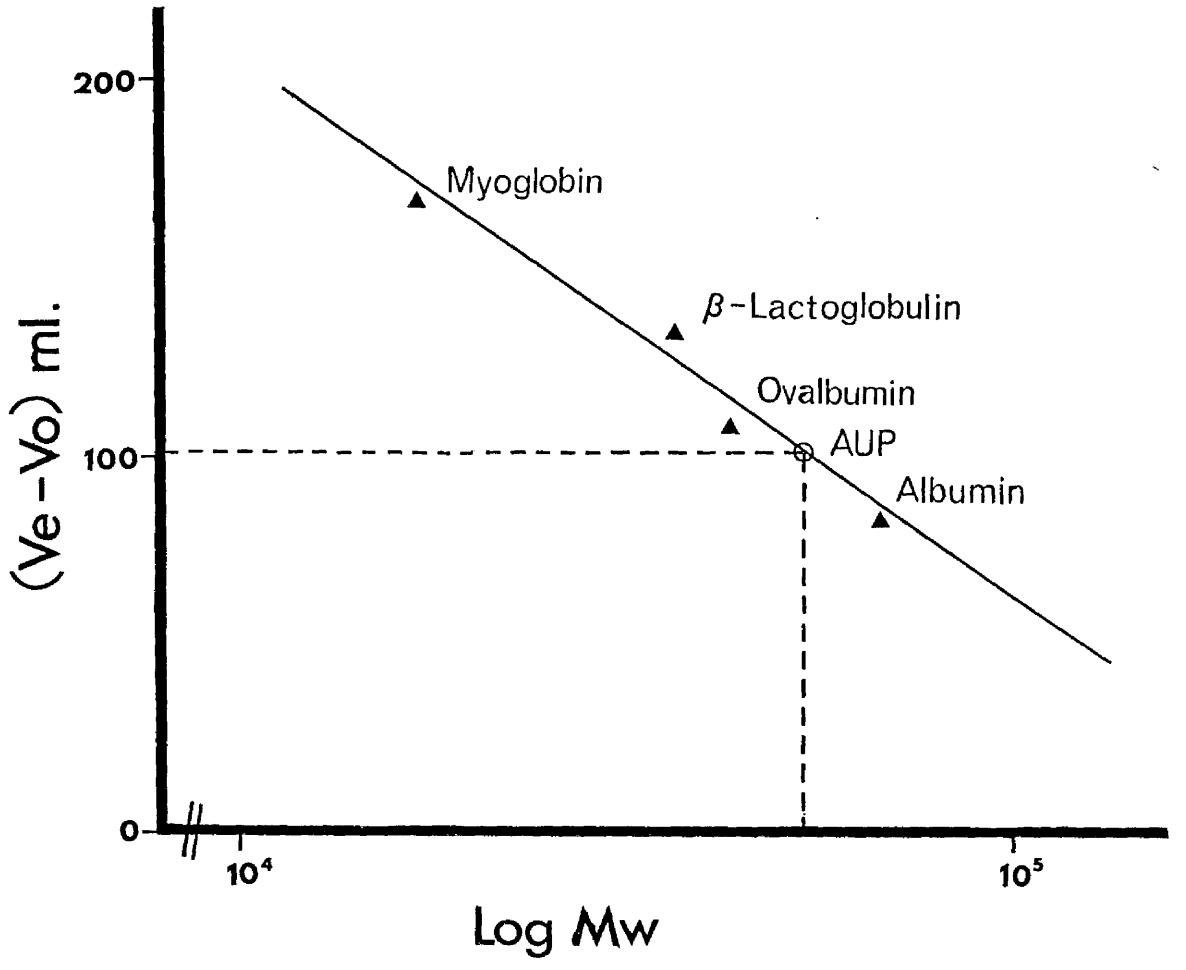


Fig. 6. Estimation of the molecular weight of AUP from a calibration graph of elution volume against Log MW.

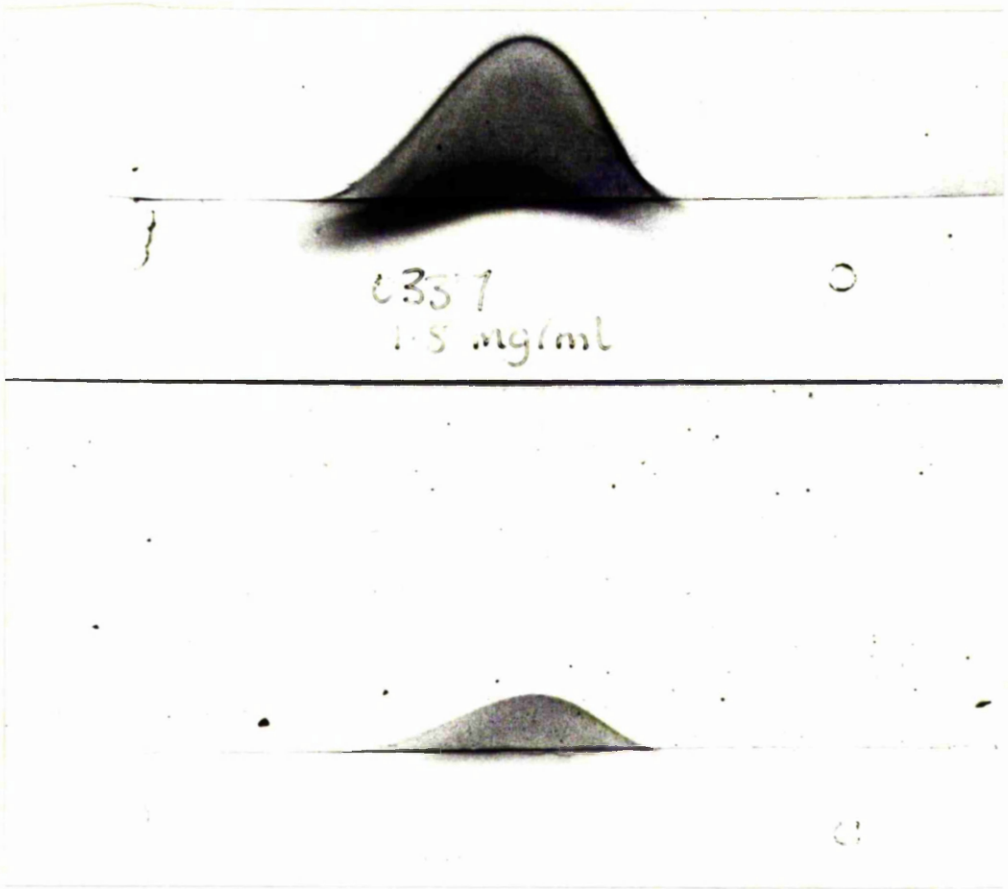


Fig. 7. Two-dimensional AACE comparing the electrophoretic mobilities of decidual and amniotic fluid AUP. The antiserum was raised against decidual AUP and adsorbed with adult male human serum. The final antiserum concentration was 20%. The antigens were inserted in wells on the left hand side of the plate and electrophoresed towards the anode (L - R) to form the first dimension. In the second dimension, each antigen was electrophoresed into a separate antibody bed.
The antigens were as follows:
Upper well: 5 µl of 11 week gestation decidua (1.8 mg/ml total protein)
Lower well: 5 µl of 14-16 week gestation amniotic fluid, pooled from various patients (2.3 mg/ml total protein).
The single circles at the RHS of the plate indicate albumin mobility in the first dimension.

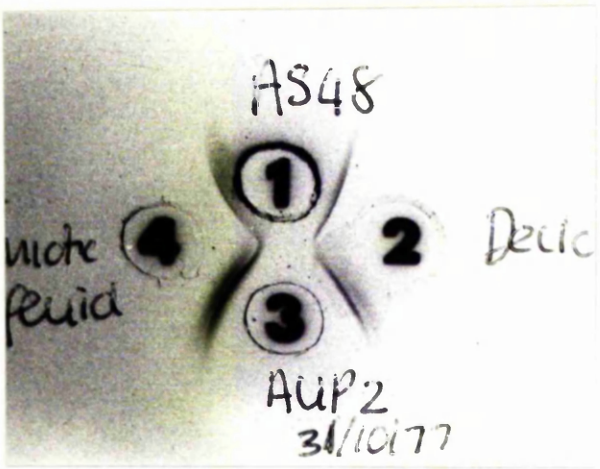


Fig. 8. Ouchterlony double immunodiffusion experiment illustrating serological identity between AUP from decidua and amniotic fluid. The wells are 4 mm in diameter. The contents of the wells are as follows:

1. rabbit antiserum to amniotic fluid AUP, adsorbed with adult human serum (10 µl).
2. homogenate of 11 week decidua at protein concentration of 0.18 mg/ml (10 µl).
3. rabbit antierum to decidual AUP, adsorbed with adult human serum (10 µl).
4. 14-16 week amniotic fluid at protein concentration of 2.3 mg/ml (10 µl).

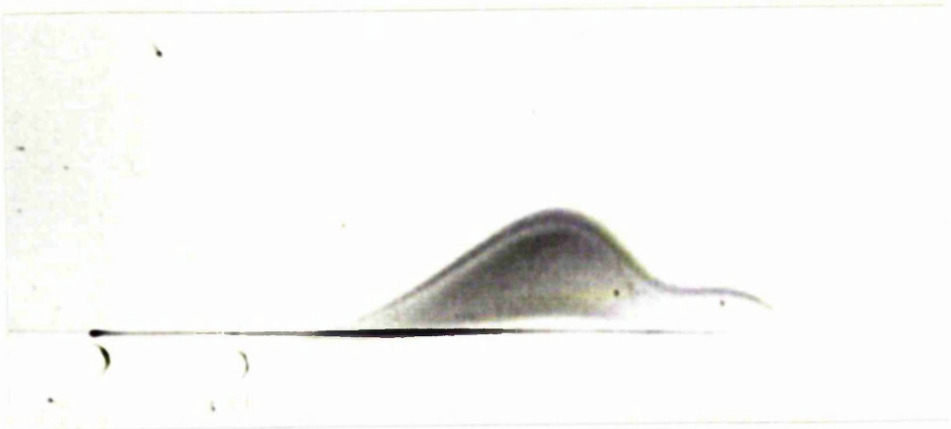


Fig. 9. Tandem-crossed electrophoresis of decidual and amniotic fluid AUP. The antiserum was raised against decidual AUP and adsorbed with adult human serum. The final antiserum concentration was 10%. The antigen wells are at the LHS of the plate. The well farthest to the left contains 2 µl of homogenate of 11 week decidua at protein concentration of 1.8 mg/ml. The other well contains 2 µl of 14-16 week amniotic fluid at protein concentration of 2.3 mg/ml.

2. Peak morphology of AUP on antibody-antigen crossed electrophoresis

Introduction: As mentioned in the preceding section, AUP produces a precipitin peak which is rather "skew" in shape on two-dimensional AACE. Another characteristic feature of the AUP peak on AACE is that it appears to be "filled-in" or solid, ie. in addition to the usual deeply-staining outline of precipitated antibody/antigen complexes the area enclosed by this peak is also more deeply-staining than the surrounding antibody bed. Several experiments were devised to investigate this phenomenon.

Methods: Using the technique of one-dimensional AACE, experiments were carried out to investigate whether any of the following variables resulted in an alteration of the characteristic AUP peak morphology:

(i) a range of antiserum concentrations from 4% to 20%.

(ii) a range of antigen concentrations from approximately 0.02-14.0 mg/ml total protein.

(iii) different sources of AUP: decidua, amniotic fluid and early gestation placenta.

(iv) antisera raised against AUP from different sources, namely decidua and amniotic fluid.

Results: The production of "filled-in" AUP peaks was not affected by the concentration of the antiserum or by the concentration or volume of the antigen. In addition, all three sources of AUP tested, namely decidua, amniotic fluid and early gestation placenta resulted in the production of such peaks. However, these peaks were only observed when AUP was reacted against antiserum raised against decidual AUP of which a total of 3 were available. When tested with the two available

antisera to amniotic fluid AUP, normal "hollow" peaks were observed (see Fig 10).

Discussion: The production of the characteristic "filled-in" AUP peak on AACE does not appear to reflect either antibody or antigen excess since varying the concentrations of each reagent had no effect on peak morphology. The results of these experiments suggest that this unusual peak morphology is a feature of antibody-antigen specificity since it occurs only with antisera raised against decidual AUP. This may indicate that, contrary to previous findings, the decidual and amniotic fluid forms of AUP may not be antigenically identical. However, the exact nature of this effect is not known.

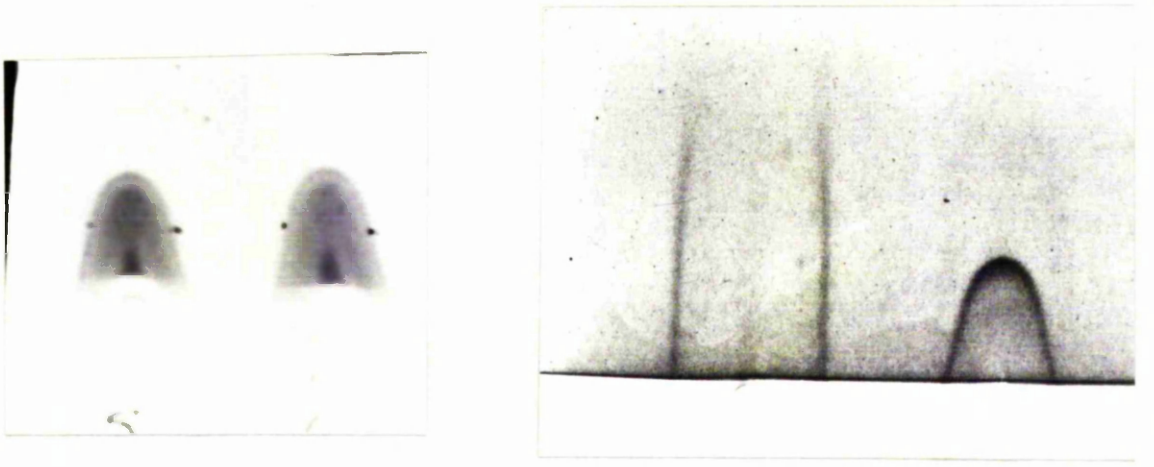


Fig. 10. One-dimensional AACE illustrating the different AUP peak morphologies obtained using antisera raised against AUP from the two major sources.

RHS. Antiserum to amniotic fluid AUP, adsorbed with adult human serum. Final antiserum concentration 20%. Antigens (L - R) : 11 week gestation decidua, at protein concentration of 0.9 mg/ml (5 µl), amniotic fluid at protein concentration of 2.3 mg/ml (5 µl).

LHS. Antiserum to decidual AUP, adsorbed with adult human serum. Final antiserum concentration 10%. Antigens (L - R): amniotic fluid at protein concentration of 2.3 mg/ml (5 µl, 6 µl).

3. The interaction between AUP and a component of Term cord serum

Introduction: Term cord serum is obtained from the umbilical cord at delivery, hence is of fetal origin. AUP cannot be detected in term cord serum (TCS) by the standard one-dimensional AACE method. However, it was noticed during routine AACE experiments checking the specificity of antisera raised against AUP that a combination of amniotic fluid and TCS resulted in the production of an AUP precipitin peak which was substantially higher than that produced by amniotic fluid alone. This effect was only observed with antiserum raised against decidual AUP from one particular rabbit, designated "AUP3". This finding suggests that there is a component in TCS which has one or more antigenic determinants in common with AUP, or may be a different protein which shares a subunit with AUP. Several experiments were carried out to further investigate this phenomenon. The aims of these experiments were as follows: a) To discover whether or not the observed effect of TCS was reproducible. b) To find if there was a similar interaction between TCS and AUP of decidual origin. c) To investigate the possibility of immunological cross-reactivity between the TCS-component and AUP. d) To investigate whether this "AUP-like" component was purely of fetal origin.

Methods: One-dimensional AACE and double immunodiffusion were carried out as described in the main "Materials and Methods" section.

Results: a) The observed effect of the addition of TCS to amniotic fluid on one-dimensional AACE resulting in an increased AUP peak height relative to that produced by amniotic fluid AUP alone was found to be

reproducible, using the same anti-AUP serum as before. This effect is illustrated in Fig 11.

b) When a combination of TCS and decidua of 11 weeks' gestation was reacted against "AUP3" antiserum on 1D AACE no increase in peak height relative to that produced by decidual AUP alone was observed.

c) A double immunodiffusion experiment was carried out to investigate whether there was any evidence of immunological cross-reactivity between AUP from amniotic fluid and the TCS-component. The wells containing TCS and amniotic fluid were placed in close proximity to determine if TCS interferes in any way with the interaction between amniotic fluid AUP and the specific antiserum. The result of this experiment is shown in Fig 12. The AUP precipitin arc extends some distance into the zone of diffusion of TCS. However, it is not clear whether this represents diffusion of AUP from the adjacent amniotic fluid well, or an interaction between the TCS-component and anti-AUP serum. Another immunodiffusion experiment was carried out to distinguish between these two possibilities. The wells were arranged as shown in Fig 13. If the observed result (Fig 12) was due to diffusion of AUP from the adjacent amniotic fluid well, the AUP precipitin line should extend an equal distance on either side of the line bisecting the amniotic fluid well (precipitin line a in Fig 13). If there is an interaction between the TCS-component and AUP, however, the precipitin line should curve round in the region of TCS diffusion (precipitin line b in Fig 13). The former result was observed (see Fig 14), indicating that there is no evidence of immunological cross-reactivity between AUP and the TCS-component on double immunodiffusion.

d) Adsorption of the "AUP3" antiserum with human term cord serum resulted in the disappearance of the TCS-component which interacted with

AUP on 1D AACE. However, there was no evidence of a similar component in any of the following fetal tissues, as investigated by 1D AACE: homogenates of fetal liver, kidney, heart, gut or skin, or fetal red cell lysates. As a further control for fetal specificity, maternal serum was combined with amniotic fluid and reacted against the "AUP3" antiserum on 1D AACE. However, the addition of maternal serum of between 9 and 41 weeks' gestation to amniotic fluid failed to produce peaks taller than those of AUP alone.

Discussion: The "AUP-like" component in TCS appears to be of fetal rather than maternal serum origin, and may be exclusive to TCS. The increased peak height on AACE resulting from the addition of TCS to amniotic fluid AUP may reflect either: (i) the attachment of the "AUP-like" component in TCS to the periphery of the existing AUP/anti-AUP insoluble complex, or (ii) the formation of soluble immunocomplexes between the TCS-component and anti-AUP antibodies, thereby reducing the number of antibodies available to react with AUP. This would result in a local decrease in antibody concentration, hence an increased peak height. As stated in the introduction, the "AUP-like" component in TCS may have one or more antigenic determinants in common with AUP, or may be a different protein which shares a subunit with AUP. This being the case, it is unclear why this component should interact with amniotic fluid AUP but not with AUP of decidual origin, particularly as the antiserum used to detect it was raised against AUP from the latter source.

Footnote: No further investigations could be done on the "AUP-like" component in TCS because the particular antiserum used to detect it, ie. "AUP3" aged, and previous results could no longer be reproduced. In addition, rabbit AUP3 subsequently died, so no further stocks of this antiserum were available.

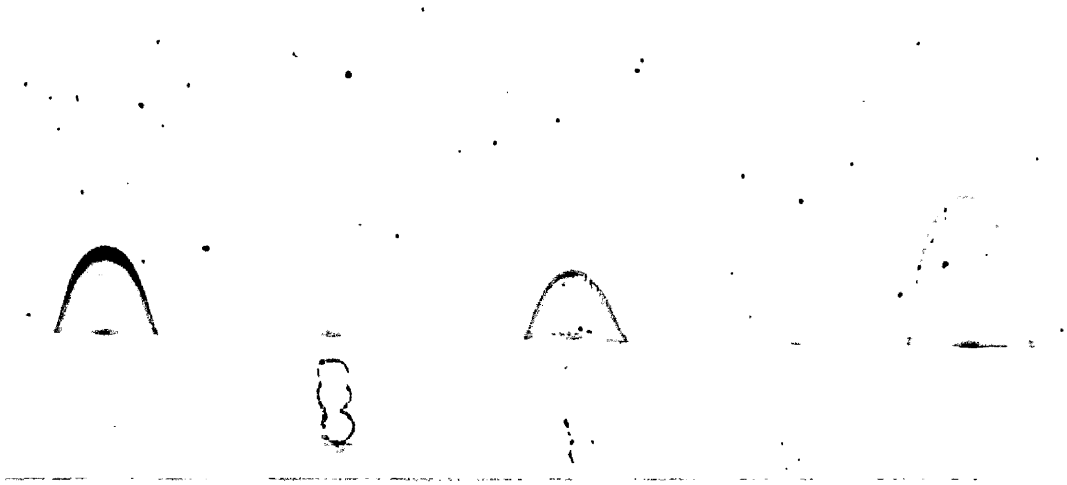


Fig. 11. One-dimensional AACE illustrating the effect of the addition of term cord serum to amniotic fluid AUP. The antiserum was raised against decidual AUP and obtained from rabbit "AUP 3". It was used in the native state at a final concentration of 20%. The antigens were as follows (L - R): 14-16 week amniotic fluid, at protein concentration of 2.3 mg/ml (5 µl), adult human serum (5 µl), amniotic fluid and adult human serum (5 + 3 µl), term cord serum (5 µl), amniotic fluid and TCS (5 + 3 µl).

Fig. 12. Double immunodiffusion experiment illustrating a possible interaction between AUP and the TCS-component. The central well contained 12 µl of "AUP 3" antiserum, used in the native state. The contents of the other wells were as follows:
Wells 1,3,5,6. 14-16 week amniotic fluid, at protein concentration of 2.3 mg/ml (10 µl).
Wells 2,4. Term cord serum (10 µl).

Fig. 13. Diagram of arrangement of wells in double immunodiffusion experiment investigating the possible interaction between AUP and an "AUP-like" component in TCS, and the possible outcomes of this experiment.

Fig. 14. Double immunodiffusion experiment illustrating the lack of cross-reactivity between AUP and the "AUP-like" component in TCS. The central well contained 12 µl of "AUP 3" antiserum (native). The other wells contained the following:
Wells 1,5. 14-16 week amniotic fluid, at protein concentration of 2.3 mg/ml (10 µl).
Wells 2,4. TCS (10 µl).
Wells 3,6. Phosphate-buffered saline (10 µl).

Fig. 12

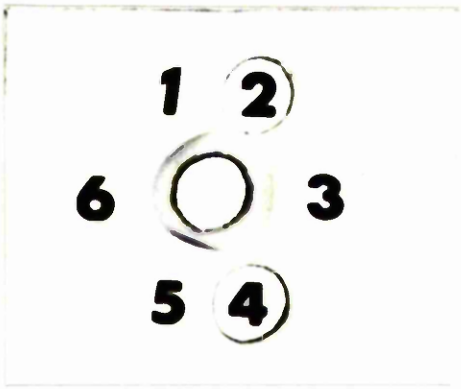


Fig. 13

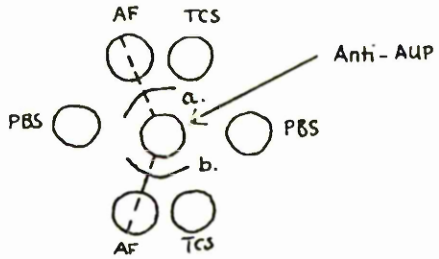
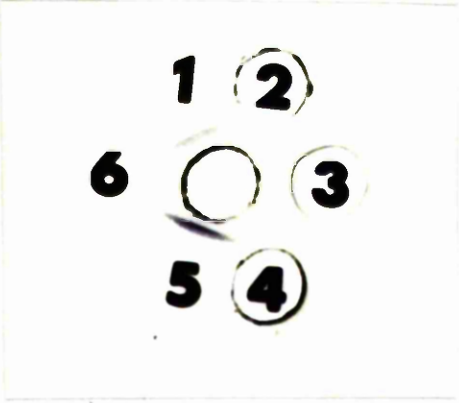


Fig. 14



4. The distribution of AUP in human adult and fetal tissues

Introduction: AUP has been detected in human endometrium, myometrium, decidua, amnion, chorion and amniotic fluid by AACE (Sutcliffe et al, 1978). It could not be detected in maternal serum, adult liver, gut, kidney, lung or skin using the same method (Sutcliffe et al, 1978, 1979). It is not yet known whether AUP is present in any fetal tissues. The possible presence of this protein in the fetus is of particular interest because AUP is present in the fetal membranes and an "AUP-like" component has been detected in term cord serum which is mainly of fetal origin. Therefore, the distribution of AUP in various fetal tissue homogenates was investigated.

Methods: The following tissues were investigated for the presence of AUP by one-dimensional AACE using antisera raised against either decidual or amniotic fluid AUP: homogenates of early gestation (8-13 weeks) placenta, term placenta, fetal gut, liver, skin, heart and 15-28 week fetal red cell lysates. Preparation of the placental homogenates is described in the following section. The homogenates of the fetal tissues and the fetal red cell lysates were previously prepared in this laboratory.

Results: AUP was detected by one-dimensional AACE in early gestation and term placentae, and in 17 week fetal gut (see Figs 15-17). In one assay, trace amounts of AUP were detected in fetal skin, but this observation could not be repeated.

Discussion: Using the technique of antibody-antigen crossed electrophoresis, AUP has now been detected in the following tissues: human endometrium, myometrium, decidua, amnion, chorion, amniotic fluid,

8-13 week placenta, term placenta, mid-gestation fetal gut and possibly fetal skin. Due to the difficulty of obtaining fetal material it is not known whether AUP is present in fetal gut at other stages of gestation. Similarly, placental material from 13 weeks of gestation to term is not readily available. However, the presence of AUP in placentae at the beginning and end of pregnancy suggests that this protein may occur in placental tissue throughout gestation. It is important to note that AUP may also occur in other fetal and maternal tissues in concentrations below the limit of detection by AACE. Therefore, in order to specify the complete tissue distribution of this protein a more sensitive technique eg. radioimmunoassay is required. It is equally important to establish the site(s) of synthesis of this protein in order to determine the origin of AUP in amniotic fluid. In addition, this information may help in elucidating the physiological function of this protein. Preliminary immunofluorescence experiments have been carried out to determine the possible site(s) of AUP synthesis in decidual and placental tissue. However, from the results of these studies it was apparent that the available antisera to AUP were of insufficiently high titre for use in immunofluorescence. Therefore, a repetition of these experiments awaits production of anti-AUP serum of high titre, which in turn is dependent upon the production of purified or partially-purified AUP.

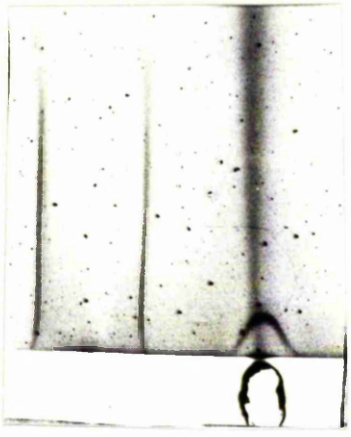


Fig. 15. One-dimensional AACE illustrating the presence of AUP in term placenta. The antiserum was raised against amniotic fluid AUP and adsorbed with adult human serum. It was used at a final concentration of 10%.

Antigens: LHS. Homogenate of 11 week decidua, at protein concentration of 0.9 mg/ml (5 μl).

RHS. Homogenate of term placenta, at protein concentration of 4.45 mg/ml (5 μl).

Fig. 16. One-dimensional AACE of AUP detected in homogenate of fetal gut. The antiserum was raised against decidual AUP and adsorbed with adult human serum. The final antiserum concentration was 10%. The antigens were, from left to right: 14-16 week amniotic fluid, at protein concentration of 2.3 mg/ml (5 µl), homogenate of fetal skin (5 µl), homogenate of 17 week fetal gut, at protein concentration of 15 mg/ml (5 µl).

Fig. 17. Double immunodiffusion experiment illustrating immunological identity between AUP in uterine decidua and fetal gut. The wells were 1.5 mm in diameter and each contained a volume of 2 µl. The contents of the wells were as follows:-
Central well: anti-AUP serum, raised against decidual AUP and adsorbed with adult human serum.
Other wells, starting at top and moving clockwise: 17 week fetal gut (15 mg/ml), 11 week decidua (1.8 mg/ml), 17 week fetal gut (15 mg/ml).

Fig. 16

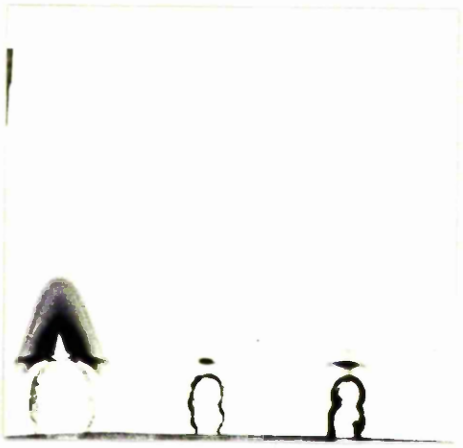
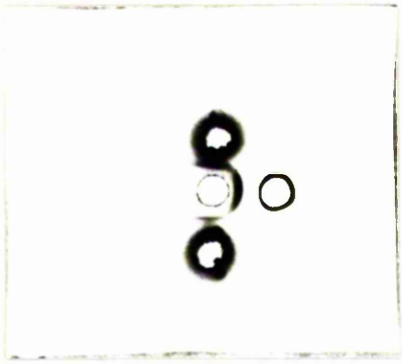


Fig. 17



5. Quantitation of AUP in uterine decidua and placenta

Introduction: The presence of AUP in homogenates of early and late gestation decidua and placenta has already been demonstrated in the preceding section, and electrophoretic and immunological identity has been established between decidual and amniotic fluid AUP (see section 1 of Results). The concentration of AUP has been measured in amniotic fluid between 12 weeks of gestation and term by one-dimensional AACE (Sutcliffe et al, 1978). High concentrations were found during the second trimester of gestation, particularly between 14 and 18 weeks. In order to determine if the concentration of AUP in uterine decidua varies in a similar manner throughout gestation, levels of AUP in homogenates of early and late gestation decidua were measured by one-dimensional AACE. Due to the availability of samples of early gestation placenta, a similar AUP quantitation was carried out in this tissue. The concentration of AUP was also measured by AACE in a total of four term placentae. Preliminary experiments were done to determine if the AUP detected in placenta was electrophoretically and immunologically identical to that in amniotic fluid and decidua.

Methods: Early gestation (8-13 weeks) decidual and placental tissue was obtained from suction terminations of pregnancy. Late gestation (35-40 weeks) decidua was obtained from elective caesarian sections. Term placentae were obtained from normal vaginal deliveries. Samples of early gestation decidua and placenta were easily distinguished by visual inspection. In some cases, identification was confirmed by microscopic examination of haemalum and eosin stained tissue sections. All tissue samples obtained were approximately 1-3 cm^2 in area and 1-3 mm thick. Each sample was washed thoroughly in PBS, blotted dry

then homogenised in an equivalent volume of PBS (about 1-5 ml, depending on the size of the tissue sample). After homogenisation, each sample was centrifuged at $4^{o}C$ (13K, 15 mins). The supernatant was drained off and filtered through Whatman No. 1 paper to remove lipids. Each homogenate was then assayed for total protein by the Lowry method. The electrophoretic mobility of placental AUP was compared to that of decidual and amniotic fluid AUP by two-dimensional AACE. Immunological cross-reactivity of AUP from these three sources was established by double immunodiffusion and one-dimensional AACE. In the latter experiment homogenates of placenta were combined with known AUP standards and reacted against specific anti-AUP serum. If the height of the resultant precipitin peak was greater than that produced by the AUP standard alone, this indicated immunological identity between placental AUP and that present in the reference standards. The concentration of AUP in each tissue homogenate was measured by one-dimensional AACE, as described in the main "Materials and Methods" section. A homogenate of 11 week gestation decidua was used as the reference standard. The results of each quantitation were expressed as "units AUP/mg protein". Each quantitation was repeated, and the average AUP concentration determined. Due to the technical difficulties involved in homogenising a complete human placenta at term an alternative means of sampling from a wide area of this organ was sought. This was achieved by excising small pieces of tissue (dimensions as described above) from different areas of the placenta, ie. from the centre and periphery, and from the fetal and maternal surfaces, as illustrated below (see Fig 18). The tissue samples were homogenised and AUP assayed by one-dimensional AACE as before.

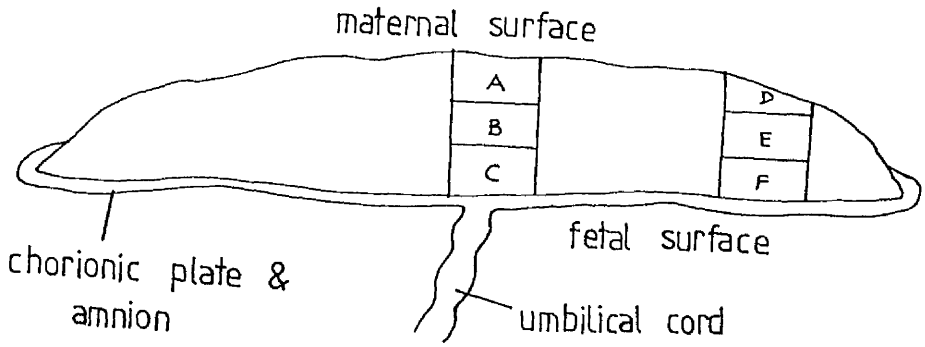


Fig. 18. Schematic drawing of term placenta indicating the areas from which tissue samples were excised. A total of six samples of placental tissue were obtained (A-F).

Results: The electrophoretic mobility of AUP in homogenates of early gestation placenta, as determined by 2D AACE, compared favourably with that of AUP of decidual and amniotic fluid origin. This is illustrated in Fig 19. Immunological identity between placental AUP and AUP from decidua and amniotic fluid was demonstrated by double immunodiffusion and 1D AACE. In the latter experiment, the combination of placenta and either decidua or amniotic fluid produced a peak which was taller than that produced by either decidua or amniotic fluid alone, when reacted against antiserum specific to AUP. The results of the quantitation of AUP in early gestation decidua and placenta and in late gestation decidua by 1D AACE are shown in Tables 3 and 4. Trace amounts of AUP were detectable in a total of 16 tissue samples of term placenta, out of the 24 samples assayed. However, AUP was present in each of the four term placentae examined, and in all six regions of the placenta sampled (A-F). No correlation was found

between the position of the tissue sample in the placenta and AUP concentration. In all cases, the concentration of AUP was too low to allow an accurate quantitation. The minimum concentration of AUP which can be accurately determined by one-dimensional AACE is 0.02 units/ml. Therefore, in the homogenates of term placentae the concentration of AUP was less than this amount. However, by taking into account the total protein concentration of each homogenate an estimate of AUP concentration, expressed as units AUP/mg protein was determined. The range and mean values of AUP concentration in the tissue samples from each placenta assayed are shown in Table 5.

Discussion: The results of the preliminary experiments indicate that placental AUP is identical to that present in decidua and amniotic fluid as regards immunological cross-reactivity and electrophoretic mobility. The results of the AUP quantitation experiments raise several interesting points, which are outlined below:

1) Early in gestation (8-13 weeks) the range of values of AUP concentration are similar in placenta and decidual tissue ie. 0.2-2.0 units/mg protein and 0.4-2.8 units/mg protein, respectively. However, the concentration of AUP is slightly higher in decidual tissue than in placenta at each stage of gestation. As previously mentioned, the presence of AUP in both pregnant and non-pregnant uterine tissue suggests that this is the major site of synthesis of this protein. However, the similarity between the levels of AUP in decidual tissue and placenta early in gestation indicates that both tissues may synthesise AUP at this stage.

2) There is no apparent correlation between gestational age and AUP concentration in either placenta or decidual tissue between 8 and 13

weeks of gestation. In a previous study (Sutcliffe et al, 1978) the concentration of AUP was measured in amniotic fluid samples between 12 weeks of gestation and term. High levels of AUP were found between 15 and 18 weeks after which there was a decline in concentration to term (see Fig 1). However, no amniotic fluid samples of less than 12 weeks' gestational age were available. Therefore, it is impossible to compare the amniotic fluid results with those of the present study. 3) In the case of amniotic fluid AUP levels there is a decline in concentration of approximately ten-fold between 12 weeks' gestation and term (Sutcliffe et al, 1978). Similarly, there is an overall decrease in AUP concentration towards term in both decidual tissue and placenta. However, in both tissues the decline in AUP concentration is very much greater than that reported for amniotic fluid AUP, ie. the values of AUP concentration in decidual tissue at delivery (see Table 4) are $\frac{1}{50}$ to $\frac{1}{100}$ of the concentrations early in gestation (see Table 3), whereas in placenta an even greater reduction in concentration of $\frac{1}{400}$ - $\frac{1}{3000}$ is found (see Tables 3 and 5).

The results of this quantitation of AUP in decidual and placental tissue are discussed more fully in the "General Discussion", with regard to the synthesis of AUP and its possible origins in amniotic fluid.

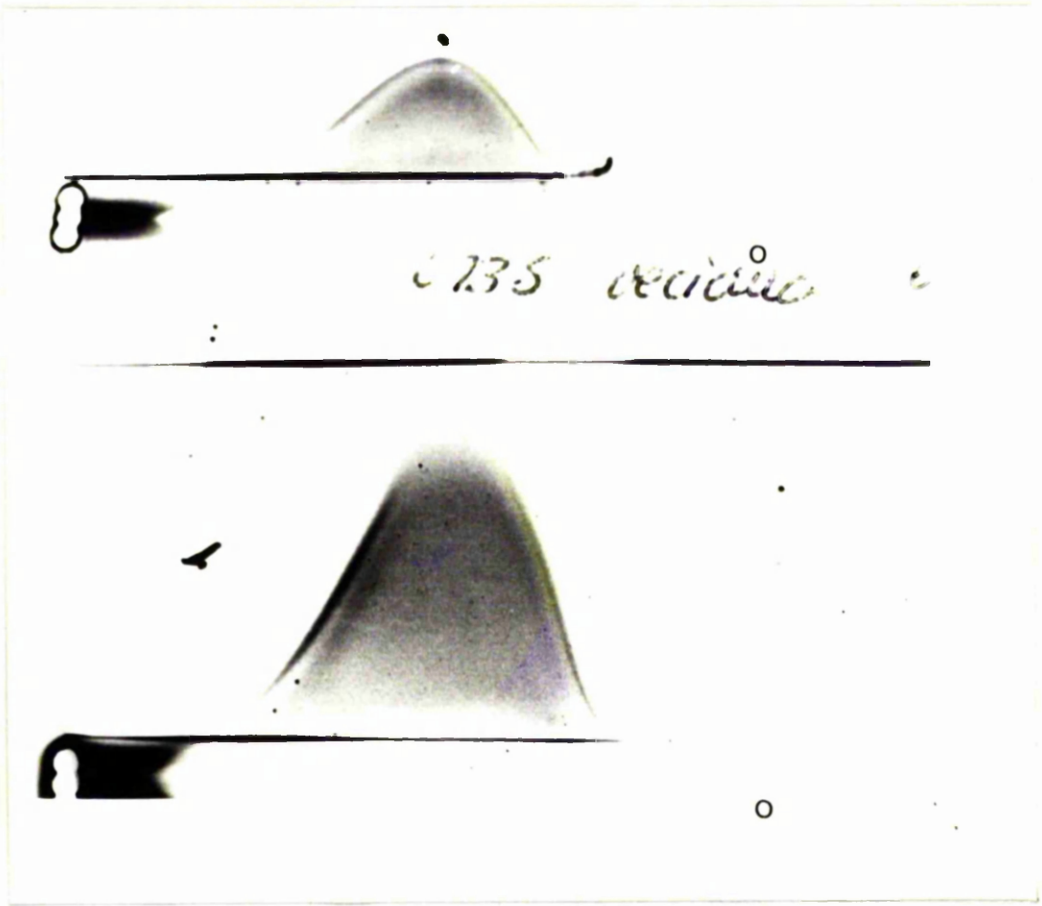


Fig. 19. Two-dimensional AACE illustrating the electrophoretic identity of AUP from decidua and placenta. The antiserum was raised against decidual AUP and adsorbed with adult human serum. The final antiserum concentration was 10%. The antigens were inserted in wells at the far LHS of the gel. Upper well: 10 week decidua, at protein concentration of 6.65 mg/ml. Lower well: 10 week placenta, at protein concentration of 32 mg/ml. The single circles at the RHS of the gel indicate albumin mobility in the 1st dimension.

Table 3. Concentration of AUP in decidual tissue and placenta early in pregnancy.

Gestation (weeks)	Conc. of AUP (units/mg protein) Decidual tissue	Placenta
8	NT[a]	0.2
8	2.8	NT
9	0.9	0.2
11	2.2	2.0
13[b]	1.4,0.4	NT
13	0.6	0.5

a not tested

b two samples of decidua were recovered from this case and assayed separately as shown.

Table 4. Concentration of AUP in decidual tissue at delivery

Gestational period (weeks)	No. of patients	AUP conc. (units/mg protein) Range	Mean
35-37	3	0.01-0.02	0.015
38-39	7	0.0025-0.085	0.027
40	4	0.004-0.01	0.0065

Table 5. Concentration of AUP in placental tissue at delivery

Gestational period (weeks)	[c]No. of tissue samples assayed per placenta	AUP conc (units/mg protein x 10^{-4}) Range	Mean
unknown	6	5.48-8.66	6.58
38	1	-	6.06
39	5	4.13-6.08	5.12
40	4	4.53-6.92	5.68

c this denotes the number of samples in which AUP was detectable by one-dimensional AACE, out of a total of 6 tissue samples assayed per placenta.

6. The partial purification of AUP from amniotic fluid

Introduction and aims: AUP has been purified from 11 week gestation decidual tissue using a three-step method of antibody affinity chromatography, ion exchange chromatography and gel filtration (Sutcliffe et al, 1980). Although this purification procedure was successful, the difficulty of obtaining decidua from early gestation precludes using this tissue for extraction of AUP on a larger scale. In contrast, samples of amniotic fluid are readily available at term, and during the second trimester of gestation from amniocenteses. The purification of AUP from amniotic fluid was therefore attempted. If this was successful, it would enable the following studies to be undertaken in the future:

1) a more detailed comparison of the structure and properties of AUP from uterine decidua and amniotic fluid.
2) the establishment of a radioimmunoassay experiment to detect trace amounts of AUP in maternal and fetal tissues thereby elucidating the complete tissue distribution of this protein.
3) the production of a higher titre antiserum to AUP for use in future experiments, eg. the localisation of AUP in maternal or fetal tissues by immunofluorescence.

Two different methods were used in an attempt to purify AUP from amniotic fluid. The first method adopted was similar to that used previously by Sutcliffe et al (1980), ie. a three-step regime of antibody affinity chromatography, ion exchange chromatography and gel filtration. A shorter two-step method, consisting of ion exchange chromatography followed by Con-A Sepharose chromatography was also tried.

a) Purification of AUP by antibody affinity chromatography, ion exchange chromatography and gel filtration

Methods: The source of AUP used in this purification procedure was pooled amniotic fluid samples of various gestations, collected by amniocentesis. The total protein concentration of this material was 4.9 mg/ml.

Antibody affinity chromatography: This step was performed on a column of Sepharose (Pharmacia) to which was coupled rabbit immunoglobulin raised against AUP from human amniotic fluid. The production of the anti-AUP serum and the preparation of the affinity column are described elsewhere (Sutcliffe et al, 1978, 1980). The bed volume of the column was approximately 90 ml. A total of nine samples of amniotic fluid were applied to the column. The binding capacity of the column was estimated by increasing the volume of the samples applied until AUP was detectable in the run through material. Each sample was applied to the affinity column then washed through with phosphate buffered saline (PBS) at 20 ml/hr at $4^{o}C$. Run through fractions were collected and monitored spectrophotometrically at 280 nm to determine total protein concentration. When the absorbance at OD280 reached background levels the remaining protein bound to the column was eluted with 50 ml of 2M potassium iodide, 50 mM Tris, pH 8.0 at 100 ml/hr at $4^{o}C$. Fractions were collected as before and monitored for total protein. The column was then rapidly washed with PBS (200 ml/hr) to remove residual KI. After about 40 minutes the flow rate was reduced to 20 ml/hr and the PBS wash was continued overnight before the next sample of amniotic fluid was applied. Run through and eluate fractions were assayed for AUP by one-dimensional AACE using anti-AUP serum. Eluate fractions found to contain AUP were pooled into a total volume

of 921 ml then dialysed against two changes, each of 5 litres, of 10 mM potassium phosphate, 50 mM NaCl, pH 7.5 for 24 hours, followed by three changes, each of 5 litres, of 10 mM potassium phosphate, pH 7.5 for 36 hours. The dialysed material was then concentrated approximately five-fold by ultrafiltration using an Amicon PM-10 membrane (exclusion limit : 10,000 daltons) to yield a final volume of 191 ml.

Ion exchange chromatography: This step was carried out on a column of DEAE-Sepharose Cl-6B (Pharmacia) of approximately 220 ml bed volume. After equilibration with 10 mM potassium phosphate, pH 7.5 at $4^{o}C$, 150 ml of the concentrated eluate from the affinity column was applied to the ion exchange column. This was washed through with 10 mM potassium phosphate, pH 7.5 at 40 ml/hr at $4^{o}C$ until the absorbance at OD280 reached background levels. A linear gradient, starting with 500 ml of 10 mM PO_4, 0.1M NaCl, pH 6.5 and ending with 500 ml of 10 mM PO_4, 0.5M NaCl, pH 6.5 at 40 ml/hr at $4^{o}C$ was then applied to the column. Both run through and eluate fractions were once again assayed for AUP by one-dimensional AACE. In addition, conductivity of the material eluted from the column by the ion exchange gradient was measured using a conductivity meter (Radiometer, Copenhagen) in order to determine the mean salt concentration at which AUP dissociates from the column. In order to further purify AUP, the ion exchange step was repeated using a longer gradient. After re-equilibration of the column with 10 mM potassium phosphate, pH 7.5, the peak AUP fractions from the first ion exchange step were pooled into a volume of 49 ml, diluted five-fold to reduce the salt concentration, then applied to the column as before. This material was washed through with 10 mM PO_4, pH 7.5

and run through fractions collected until the OD280 fell to background levels. The second linear gradient was then applied to the column, starting with one litre of 10 mM PO_4, 0.05M NaCl, pH 6.5 and ending with one litre of 10 mM PO_4, 0.2M NaCl, pH 6.5, at 40 ml/hr at 4°C. Both run through and eluate fractions were assayed for AUP by 1D AACE. In addition, the eluted material was assayed for albumin by 1D AACE using anti-human albumin serum (Behningwerke).

Gel filtration: The peak AUP fractions eluted from the second ion exchange run were pooled and concentrated by ultrafiltration to a volume of 12.5 ml. This material was further concentrated to a final volume of 6 ml by lyophilisation, weighted with glycerol then applied to a 3.5 x 90 cm column of Sephadex G-150 (Pharmacia). The column was washed with PBS at 10 ml/hr at 4°C and fractions collected and assayed for AUP and albumin by 1D AACE as before.

Results and Discussion

Affinity chromatography: Volumes of amniotic fluid ranging from 15-100 ml were applied to the antibody affinity column. When samples of 50 ml and 100 ml were applied, AUP was detectable in run through fractions in concentrations measurable by AACE. No AUP was detectable in the run through when the sample volume was 15 ml, whereas AUP was just detectable in the run through by AACE when 20 ml amniotic fluid was applied to the column. During the course of the purification, the amniotic fluid pool used as the starting material was also used as a reference standard in quantitations of AUP by AACE, and as such was arbitrarily said to have an AUP concentration of 1 unit/ml. Thus, the column capacity was approximately 20 "units" of AUP. The AUP recovery data from the nine separate runs of the column is summarised

in Table 6. The overall recovery of AUP from the antibody affinity chromatography step was 39%. An identical recovery from this step was reported by Sutcliffe et al (1980) during the purification of AUP from uterine decidua.

Ion exchange chromatography: A volume of 150 ml of the pooled material eluted from the affinity column was applied to the column of DEAE-Sepharose Cl-6B. This material contained approximately 57 mg protein and 0.52 units AUP/ml, therefore a total of 78 "units" of AUP were applied. The protein profile of the material eluted with the first gradient of 0.1-0.5M NaCl is shown in Fig 20. No AUP was detectable in the run through fractions by one-dimensional AACE, but this protein was found in a total of 18 x 10 ml eluate fractions (nos. 7-24 on Fig 20) by this method. Fraction nos. 7, 8 and 9 contained maximal levels of AUP. On inspection of the protein profile, it was evident that AUP was eluted in three protein peaks, and that the third peak which contained the maximum AUP concentration merged with another major protein peak. From a standard curve of conductivity against molarity (Fig 21), it was estimated that AUP was eluted at salt concentrations ranging from 0.045-0.20M. The major contaminant is likely to be serum albumin, because this dissociates from DEAE-Sepharose at a mean salt concentration of 0.18M NaCl (Sutcliffe et al, 1980). In order to determine more accurately the mean salt concentration required to dissociate AUP from the column and to separate AUP from the major contaminant protein(s) visualised in Fig 20, the ion exchange step was repeated using a longer gradient of 0.05-0.2M NaCl, as described in the Materials and Methods section. Six fractions (nos. 14-19) from the first ion exchange step containing high levels of AUP were pooled into

a volume of 49 ml. This pooled material, which contained 0.19 mg/ml total protein and 1.13 units AUP/ml (as estimated by 1D AACE), was applied to the column and run through and eluate fractions collected as before. However, when these fractions were monitored for absorbance at OD280, only trace amounts of protein were found. AUP was not detectable in the run through fractions by 1D AACE but was detected in a total of 36 eluate fractions by this method. No albumin was detected in these 36 fractions by 1D AACE. Examination of the conductivity measurements of the eluate fractions revealed that the conductivity rose initially then reached a plateau, which suggests that the ion exchange gradient was insufficiently mixed during the course of the experiment. In Fig 22 the conductivity profiles of the material eluted during the first and second runs of the ion exchange column are compared. The eluate fractions from the second ion exchange run, containing maximal levels of AUP as determined by 1D AACE were pooled into a volume of 194 ml. This was concentrated by ultrafiltration to a final volume of 12.5 ml. The concentrated material contained 0.55 mg protein and 0.24 units AUP/ml. The recovery of AUP from the two ion exchange steps is summarised below:

Starting material: pooled material from affinity chromatography step.

Volume : 150 ml

AUP concentration : 0.52 units/ml

Total AUP : 78 units

↓

1st ion exchange step

↓

1st ion exchange eluate (pooled fractions 14-19)

Volume : 49 ml

AUP concentration : 1.13 units/ml

Total AUP : 55.37 units

∴ Recovery of AUP after 1st ion exchange step was

approximately 71%

↓

2nd ion exchange step

↓

2nd ion exchange eluate

Volume : 12.5 ml

AUP concentration : 0.24 units/ml

Total AUP : 3 units

∴ Recovery of AUP after 2nd ion exchange step was 5.4%

It is evident that there was a much reduced recovery of AUP from the second ion exchange step. Measurement of the conductivity of the material eluted by the second ion exchange gradient revealed that the maximum conductivity corresponded to a salt concentration of 0.115M NaCl, and that the fractions containing AUP were eluted at salt concentrations ranging from 0.075-0.115M NaCl. It was previously reported that AUP dissociates from DEAE-Sepharose at a mean salt concentration of 0.13M NaCl (Sutcliffe et al, 1980). Therefore, it is possible that the majority of the AUP applied to the column remained bound because the ionic strength of the eluting gradient was insufficiently high to effect dissociation. However, application of another gradient of increased ionic strength, starting with 950 ml of 10 mM PO_4, 0.05M NaCl, pH 6.5 and ending with 950 ml of 10 mM PO_4, 0.3M NaCl, pH 6.5 failed to recover any AUP which may have been adsorbed on to the column. An alternative hypothesis which may account for the low recovery of AUP from the second ion exchange step is that denaturation of AUP occurred during the extensive purification procedure.

Gel filtration: The material containing AUP recovered from the second ion exchange step was further concentrated by lyophilisation to a final volume of 6 ml and applied to a column of Sephadex G-150, as described in the Materials and Methods section. This gel filtration step was carried out in an attempt to recover a small (μg) amount of AUP. However, no AUP was detectable in any of the one ml fractions collected. The possible reasons for this failure to recover any AUP from the gel filtration step are as follows:

1) The large dimensions of the column used (3.5 x 90 cm) may have resulted in extreme dilution of AUP, to the extent that the concentration in the fractions collected was below the limit of detection by AACE.

2) Denaturation of AUP may have occurred during gel filtration.

3) AUP may have adsorbed to the column of Sephadex G-150 under the experimental conditions employed. This is unlikely, however, because similar conditions of temperature, buffer etc were used in a previous gel filtration experiment, using the same column of Sephadex G-150, with successful results. (See section 1 of "Results").

Table 6. The recovery of AUP from antibody affinity chromatography during the three-step purification procedure

Material	Volume ml	(AUP) units/ml	AUP units (X)	Protein mg (Y)	AUP/protein units/mg (X/Y)
Applied to column	360	1	360	1764	0.20
Not retained by column	791.5	0.13	104.11	1458.2	0.07
∴ retained prior to elution	-	-	255.89	305.8	0.84
Eluted in 2M KI, 50 mM Tris	191	0.52	99.84	72.85	1.37
% recovery			39%		

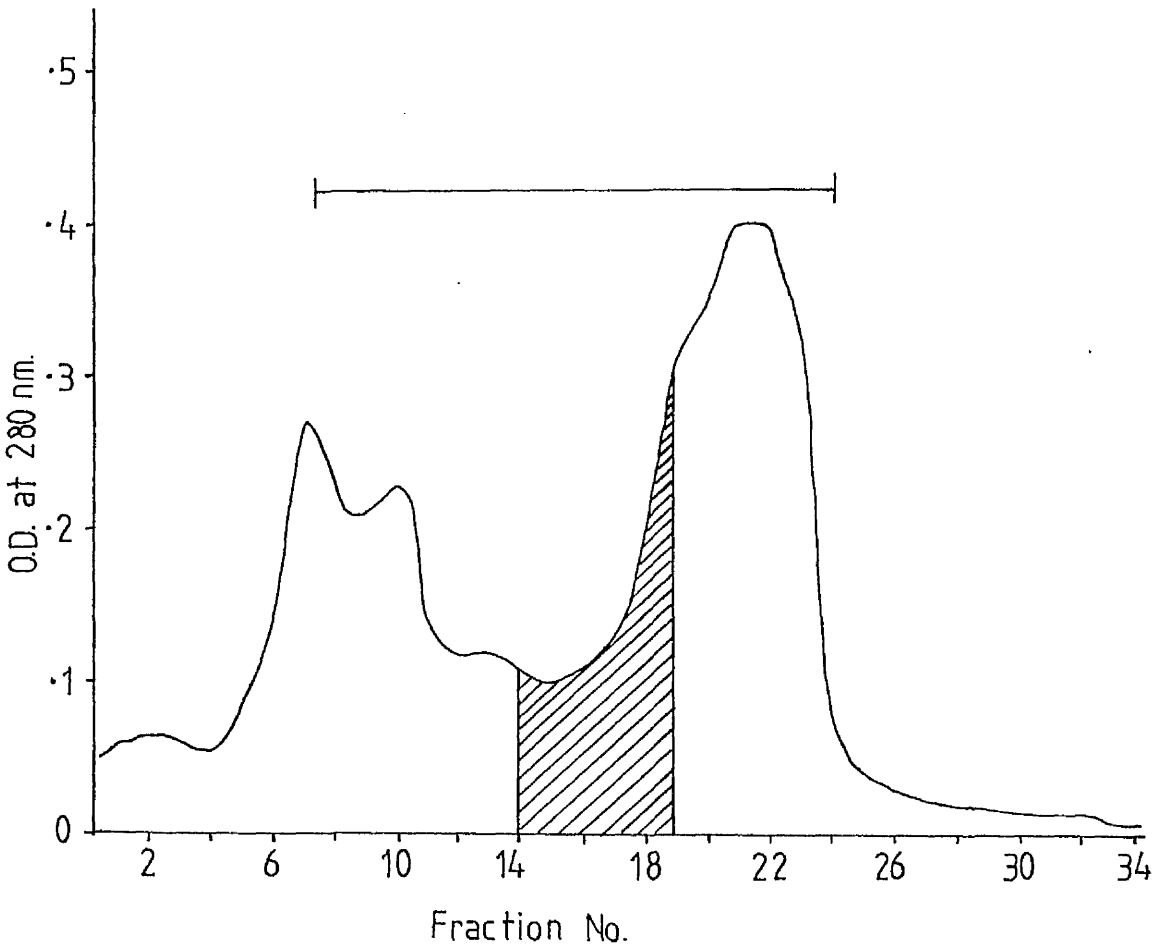


Fig. 20. Spectrophotometric trace of protein eluted from a DEAE-Sepharose Cl-6B column with an ion exchange gradient of 0.1-0.5M NaCl. Horizontal line indicates fractions containing AUP. Shaded area indicates fractions pooled.

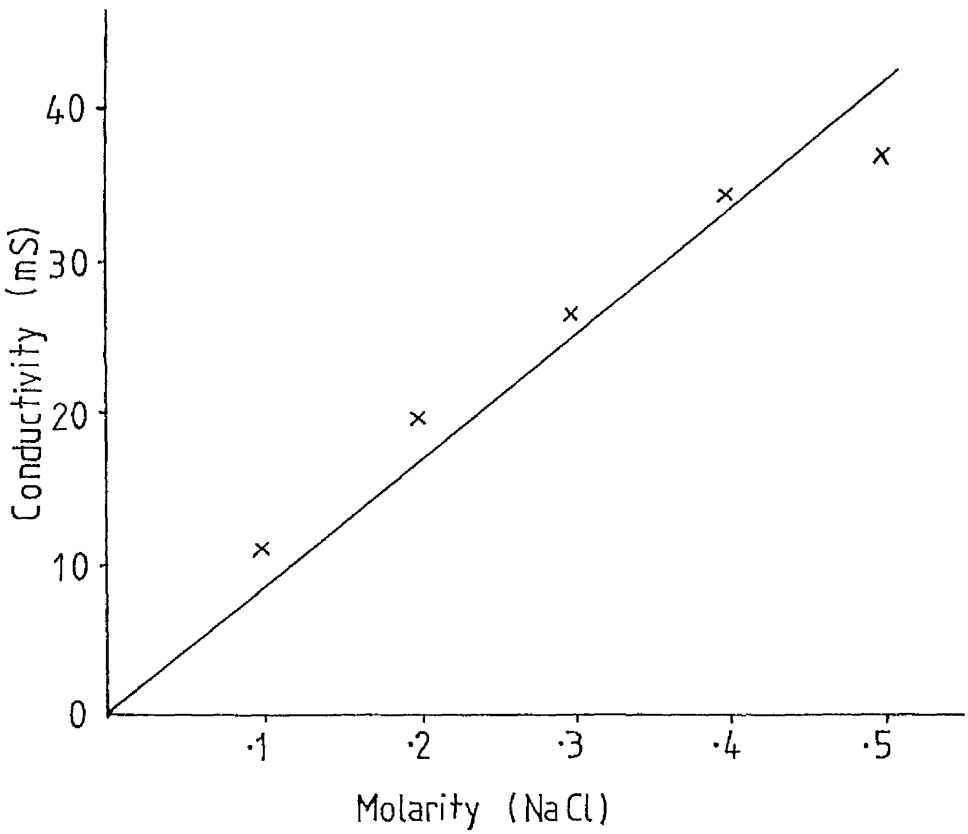


Fig. 21. Standard curve of conductivity versus molarity of NaCl.

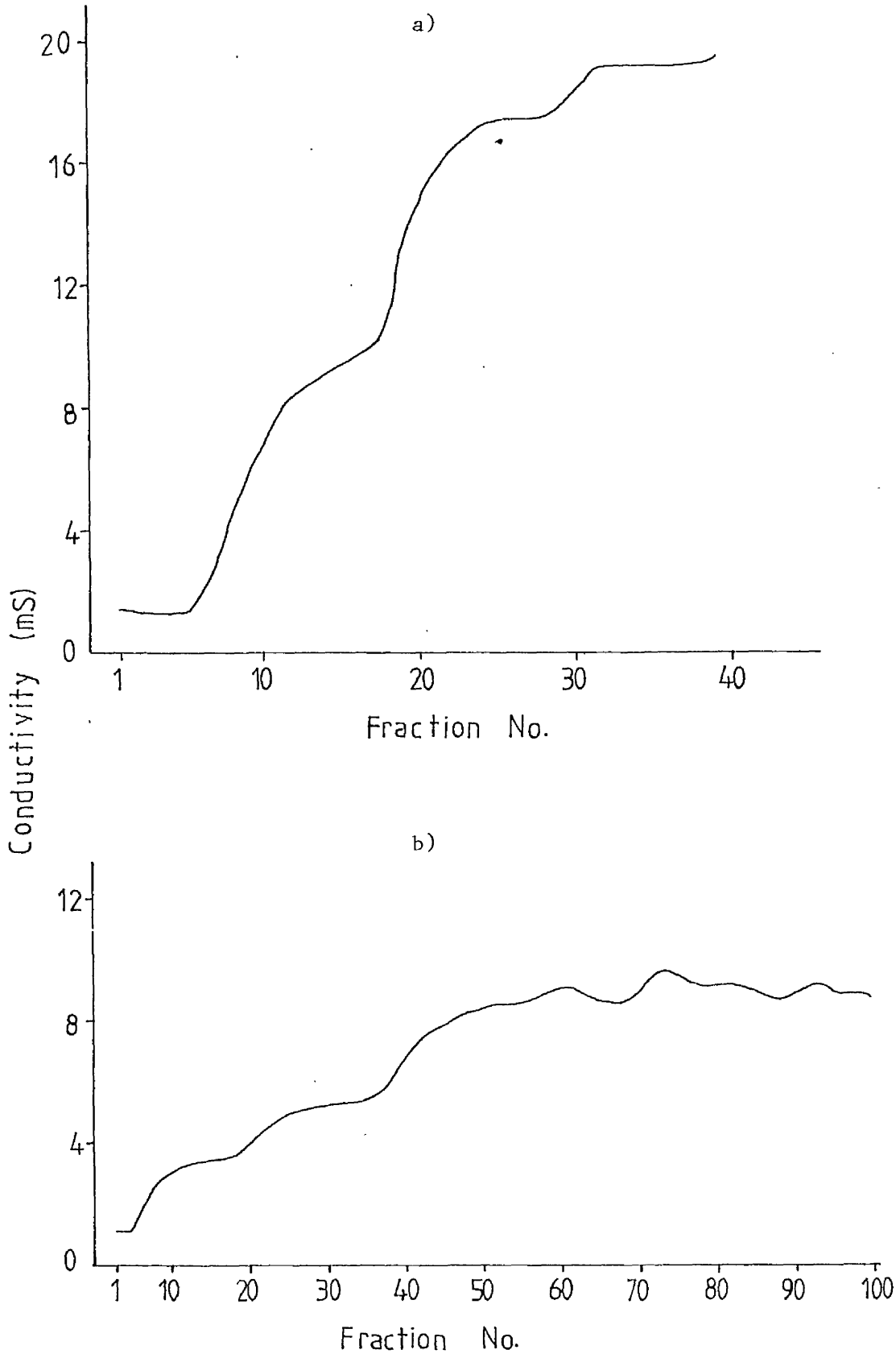


Fig. 22. Comparison of the conductivity profiles of the ion exchange eluates from the first and second gradients, in the three-step purification procedure. a) 1st ion exchange step. Gradient: 500 ml 10 mM PO_4, 0.1M NaCl, pH 6.5 - 500 ml 10 mM PO_4, 0.5m NaCl, pH 6.5. b) 2nd ion exchange step. Gradient: 1 litre 10 mM PO_4, 0.05M NaCl, pH 6.5 - 1 litre 10 mM PO_4, 0.2M NaCl, pH 6.5.

b) Purification of AUP by ion exchange chromatography and Concanavalin-A Sepharose chromatography

This two-step method of purification was adopted in an attempt to minimise the technical problems encountered during the first procedure. In particular, an alternative step to gel filtration, which may cause dilution problems was sought. It is possible that AUP is a glycoprotein, although there is as yet no unequivocal evidence to support this theory. For this reason, the method of Con-A Sepharose adsorption was utilised.

Methods:

Ion exchange chromatography: Pooled amniotic fluid of 16-22 weeks' gestation and protein concentration 5.2 mg/ml was filtered through Whatman No. 1 paper to remove particulate material then dialysed overnight against 10 mM potassium phosphate, pH 7.5. This starting material was used as the reference standard in AUP quantitations throughout the purification procedure, hence was arbitrarily said to have an AUP concentration of 1 unit AUP/ml. The column of DEAE-Sepharose Cl-6B used in the preceding experiment was re-equilibrated with 10 mM PO_4, pH 7.5, at $4^{o}C$. The ion exchange step was performed twice: in the first run, 75 ml of amniotic fluid was applied and in the second run 100 ml of amniotic fluid. The experimental conditions of temperature, buffers, flow rate etc. were constant during each run of the column. Each amniotic fluid sample was applied to the column and washed through with 10 mM PO_4, pH 7.5 at 40 ml/hr, $4^{o}C$, until the absorbance at OD280 reached background levels. A linear gradient, starting with 750 ml of 10 mM PO_4, 0.05M NaCl, pH 6.5 and ending with 750 ml of 10 mM PO_4, 0.3M NaCl, pH 6.5 at 40 ml/hr, $4^{o}C$ was then applied. Finally, the column

was washed with approximately one bed volume of the highest ionic strength buffer, 10 mM PO_4, 0.3M NaCl, pH 6.5. The protein concentration profile (absorbance at OD280) and conductivity of the eluted material were monitored. Both run through and eluate fractions were assayed for the presence of AUP by 1D AACE as before. Eluate fractions containing AUP from each run of the column were pooled into four separate batches according to AUP concentration and the extent of serum protein contamination. Each pool was then concentrated by ultrafiltration using an Amicon PM10 membrane and assayed for total protein concentration using the Folin reaction, and AUP concentration by 1D AACE.

Concanavalin-A Sepharose Chromatography: Two preliminary experiments were carried out to determine the suitability of this technique for AUP purification. These were as follows:-

1) Dialysis of amniotic fluid against Pagé buffer. This buffer contains magnesium and calcium ions which are essential components in the binding reaction between Concanavalin-A and glycoproteins. This experiment was carried out in order to determine whether exposure to Pagé buffer caused denaturation of AUP. A 15 ml volume of amniotic fluid (pooled from various gestations), of protein concentration 5·6 mg/ml was filtered through Whatman No. 1 paper to remove particulate material then dialysed overnight against 2 litres of Pagé buffer at 4°C, followed by 2 litres of 10 mM potassium phosphate, pH 7.5 at 4°C. An aliquot of amniotic fluid was removed after each stage of dialysis and assayed for AUP by 1D AACE. Protein denaturation results in an alteration of the normal peak morphology on AACE.

2) Con-A Sepharose chromatography of crude amniotic fluid. This experiment was performed to determine whether or not AUP binds to

Concanavalin-A. A column of 1 x 15 cm (K9, Pharmacia) was used in this, and subsequent Con-A Sepharose adsorption experiments. The column comprised a basal layer of approximately 0.5 cm depth of Sephadex G-25 (Pharmacia) and an upper layer of 5 ml Con-A-Sepharose (Pharmacia), previously equilibrated with Pagé buffer. Ten ml of amniotic fluid, of protein concentration 5.33 mg/ml was dialysed against Pagé buffer then applied to this column. The sample was washed through with the same buffer at 32 ml/h and run through fractions collected. Unadsorbed protein was monitored by Uvicord (LKB), which measures absorbance at OD254, and washing was continued until the protein in the run through declined to background levels. The column was then eluted with 50 mM methyl-α-D-glucopyranoside at 32 ml/hr until a second protein peak was observed on the Uvicord trace. When no more protein was detectable in the eluate, the column was re-equilibrated with Pagé buffer. All fractions were assayed for AUP and serum proteins by 1D AACE. Eluate fractions containing the maximum amount of AUP were pooled and assayed for protein and AUP concentration. In addition, the extent of serum protein contamination in this material was estimated by two-dimensional AACE, using antiserum raised in sheep against human serum proteins.

Con-A Sepharose chromatography of partially-purified AUP

After these preliminary experiments, the Con-A Sepharose chromatography step was repeated using partially-purified AUP obtained from the preceding ion exchange step. The experimental procedure used was identical to that described above, with two exceptions. Firstly, the flow rate was reduced to 28 ml/hr, and secondly, the eluting buffer used was 1M methyl-α-D-glucopyranoside. Two separate adsorption

experiments were carried out. In the initial experiment, the starting material was the peak AUP cut (pooled fractions 88-97) from the first ion exchange run. In a further experiment, three batches of ion exchange eluate fractions containing AUP: nos 68-87 and 98-105 from the 1st ion exchange run and nos. 71-93 from the 2nd ion exchange run were pooled and applied to the Con-A Sepharose column.

Results and Discussion

Ion exchange chromatography: No AUP was detectable in the run through fractions by one-dimensional AACE. However, AUP was detected by this method in a total of 73 eluate fractions (nos. 68-105) from the first run of the column and in 69 eluate fractions (nos. 65-109) from the second ion exchange run. The volume of each fraction was approximately 8 ml. Thus it was apparent that AUP did not elute from the column as a sharp peak of protein, but a gradual dissociation occurred as the ionic strength of the eluting buffer increased. Fig 23 shows the protein concentration and conductivity profiles of the material eluted by the ion exchange gradient during the first run of the column. A similar protein and conductivity profile was obtained from the second ion exchange run. From the conductivity measurements of the eluate fractions containing maximal levels of AUP (as measured by 1D AACE) in each ion exchange run, it was estimated that AUP eluted at a mean salt concentration of 0.125M NaCl during the first run and at 0.14M NaCl during the second run. These results compare favourably with the value of 0.13M NaCl reported by Sutcliffe et al (1980). From inspection of the data on protein concentration changes and AUP concentration changes in the eluate fractions measured by 1D AACE, fractions containing AUP from each ion exchange run were combined into three separate pools

(as indicated by the shaded areas in Fig 23). Since the majority of protein present in amniotic fluid is of serum origin (see main "Introduction"), the total protein present reflects the degree of serum protein contamination in the eluate fractions. Thus, in each case, "pool 1" contained a relatively low concentration of both AUP and serum proteins, "pool 2" contained the maximal AUP concentration but a higher degree of serum protein contamination, and "pool 3" contained a relatively high AUP concentration but was extensively contaminated with serum proteins. Each pool was concentrated by ultrafiltration using an Amicon PM-10 membrane then assayed for total protein and AUP concentrations. The results are shown in Table 7. The recovery of AUP from the ion exchange step could not be estimated because the amount of protein which remained bound to the column after the run through step was not measured. However, the amniotic fluid applied to the column had an AUP concentration of 1 unit/ml or 0.19 units/mg protein. Thus it was apparent that there was a considerable enrichment of AUP after the ion exchange step, particularly in pooled fractions 88-97 from the first run which contained 8·6 units AUP/mg protein, ie. about 45 times the concentration of AUP in the starting material. The second ion exchange run was less successful in that there was a much lesser enrichment of AUP and more serum protein contamination. This probably reflects the increased loading of the column: 100 ml amniotic fluid containing 520 mg protein was applied compared with 75 ml amniotic fluid containing 390 mg protein in the first ion exchange run.

On the basis of the results shown in Table 7, pooled fractions 88-97 from the first ion exchange run were used as the starting material for the next stage of the purification procedure because, out of the six ion exchange pools, this pool contained the maximum concentration of AUP

per mg protein, combined with the minimum total protein concentration.

Con-A Sepharose chromatography:

1) Dialysis of amniotic fluid against Pagé buffer. No difference was observed in the morphology of the AUP precipitin peak on 1D AACE after dialysis of amniotic fluid against Pagé buffer. It was therefore concluded that exposure to Pagé buffer does not cause denaturation of AUP.

2) Con-A Sepharose chromatography of crude amniotic fluid. From the Uvicord protein trace (absorbance at OD254) of the fractions obtained from the column after application of 10 ml amniotic fluid it was apparent that there was a much greater amount of protein in the run through fractions (unbound protein) than in the eluate fractions (protein bound to the column and dissociated with methyl-α-D-glucopyranoside). In addition, protein eluted from the column as a sharp, discrete peak. Elution commenced at fraction no. 41. Low concentrations of AUP were detectable by 1D AACE in a total of seven run through fractions (nos. 16-22), which indicates that the column was overloaded. Higher concentrations of AUP were found in eluate fractions 47-57 by this method. This suggests that AUP adsorbed to Con-A and was subsequently eluted with methyl-α-D-glucopyranoside. Serum proteins, predominantly albumin were present in all fractions assayed. However, the albumin concentration was lower on the eluate than in the run through fractions and declined as elution progressed. This suggests that albumin does not bind specifically to Con-A Sepharose, but is present in the eluate due to insufficient washing of the column to remove unadsorbed protein prior to the elution step. A total of six eluate fractions, containing maximal concentrations of AUP were pooled into a volume of 9 ml. The concentrations of protein and AUP in this pool were measured in order

to estimate the degree of enrichment of AUP after Con-A Sepharose chromatography (see Table 8). From these results it was apparent that the concentration of AUP per mg protein was more than 3-fold greater in the eluted material than in the starting material. Recovery of AUP after this step could not be accurately determined due to overloading of the column.

The results of this preliminary study indicated that AUP bound to Con-A Sepharose and was eluted with methyl-α-D-glucopyranoside and that during this procedure a 3-fold enrichment of AUP occurred. It was therefore concluded that Con-A Sepharose chromatography was a suitable second step in the purification procedure.

Con-A Sepharose chromatography of partially-purified AUP.

In the initial experiment, 9 ml of pooled ion exchange fractions 88-97 (from the first ion exchange run) was applied to the column. This material contained 2.43 mg protein and approximately 21 units of AUP. However, after Con-A Sepharose chromatography only trace amounts of AUP were detected by 1D AACE in the eluate. The most likely explanation for this poor recovery of AUP is that an insufficient quantity of protein was applied to the column, with the result that the concentration of AUP in the resultant fractions was lowered by dilution. Therefore, a second experiment was carried out, in which a greater amount of protein was loaded on the column. Fraction nos. 68-87 and 98-105 from the first ion exchange run and nos. 71-93 from the second ion exchange run were combined into a single pool, of volume 33.3 ml. Of this, 28 ml was applied to the Con-A Sepharose column. The run through and elution stages were carried out as before and fractions collected. The protein profile of the resultant fractions is illustrated

in Fig 24. Elution commenced at fraction no. 50. Trace amounts of AUP were detectable by 1D AACE in run through fractions 25-35, whereas much greater concentrations of AUP were detected in eluate fractions 56-70 by this method. As the run through step progressed, the concentration of albumin (as measured by 1D AACE) diminished until a constant low level was reached. In addition, both the concentration and number of other serum components decreased (see Fig 25a). On elution with glucopyranoside however, an increased number of serum contaminants was observed, indicating that several serum glycoproteins were present in the starting material applied to the column. However, minimal concentrations of albumin, the quantitatively major serum contaminant, were present in the eluted material (see Fig 25b). A total of seven eluate fractions (nos. 62-68) which contained relatively high concentrations of AUP but relatively low levels of serum proteins were pooled into a final volume of 8 ml. The recovery of AUP in these fractions is shown in Table 9. Note that although AUP was detectable in several run through fractions, the concentration of AUP in this material was too low to be accurately measured by 1D AACE. Thus, almost all the AUP applied to the column adsorbed to Con-A. Although the recovery of AUP was relatively low, ie. about one-fifth of the AUP applied to the column was recovered in fractions 62-68, AUP was detected in several other eluate fractions not included in this pool. Thus the overall recovery of AUP after Con-A Sepharose chromatography was somewhat higher than this value. This step also resulted in further enrichment of AUP by about two-fold, as outlined below:

Summary of purification of AUP by two-step method.

Starting material : amniotic fluid

AUP concentration : 0.19 units AUP/mg protein

↓

Ion exchange chromatography

↓

Pooled ion exchange fractions from 1st and 2nd runs of column

AUP concentration : 2.37 units AUP/mg protein

∴ There was a 12.5-fold enrichment of AUP after ion exchange

↓

Con-A Sepharose chromatography

↓

Pooled eluate fractions (nos. 62-68)

AUP concentration : 4.03 units AUP/mg protein

∴ There was a 1.7-fold enrichment of AUP after Con-A Sepharose chromatography.

Therefore after the two purification stages the concentration of AUP increased by approximately 21-fold. In order to estimate the degree of serum protein contamination in the pooled fractions (62-68) eluted from the Con-A Sepharose column this material was analysed by two-dimensional AACE using antiserum raised against adult human serum. The results were compared with those of a corresponding assay of the material applied to the Con-A Sepharose column. The 2D AACE was semi-quantitative in that the same amount of AUP (μunits) was applied to each plate. The results are shown in Fig 26. The major serum protein detected was albumin. For a given protein, the area enclosed by the precipitin peak on AACE is proportional to its concentration. Thus, a rough estimate of the reduction in albumin concentration in the partially-purified material obtained after the Con-A Sepharose step was obtained by comparing the areas under the albumin peaks on each

plate. The concentration of albumin in the starting material (pooled ion exchange fractions 68-87 + 98-105 + 71-93) was found to be approximately 12 times that in the material eluted from the Con-A Sepharose column.

Therefore, the two-step purification procedure devised was more successful for the purification of AUP from amniotic fluid than the three-step procedure used previously by Sutcliffe et al (1980). However, due to the relatively low amount of partially-purified AUP obtained from the two-step purification, no further purification was attempted.

Fig. 23. Total protein and conductivity profiles of amniotic fluid components eluted from DEAE-Sepharose Cl-6B using a linear gradient of 750 ml 10 mM PO_4, 0.05M NaCl, pH 6.5 to 750 ml 10 mM PO_4, 0.3M NaCl, pH 6.5. The shaded areas represent fractions pooled.

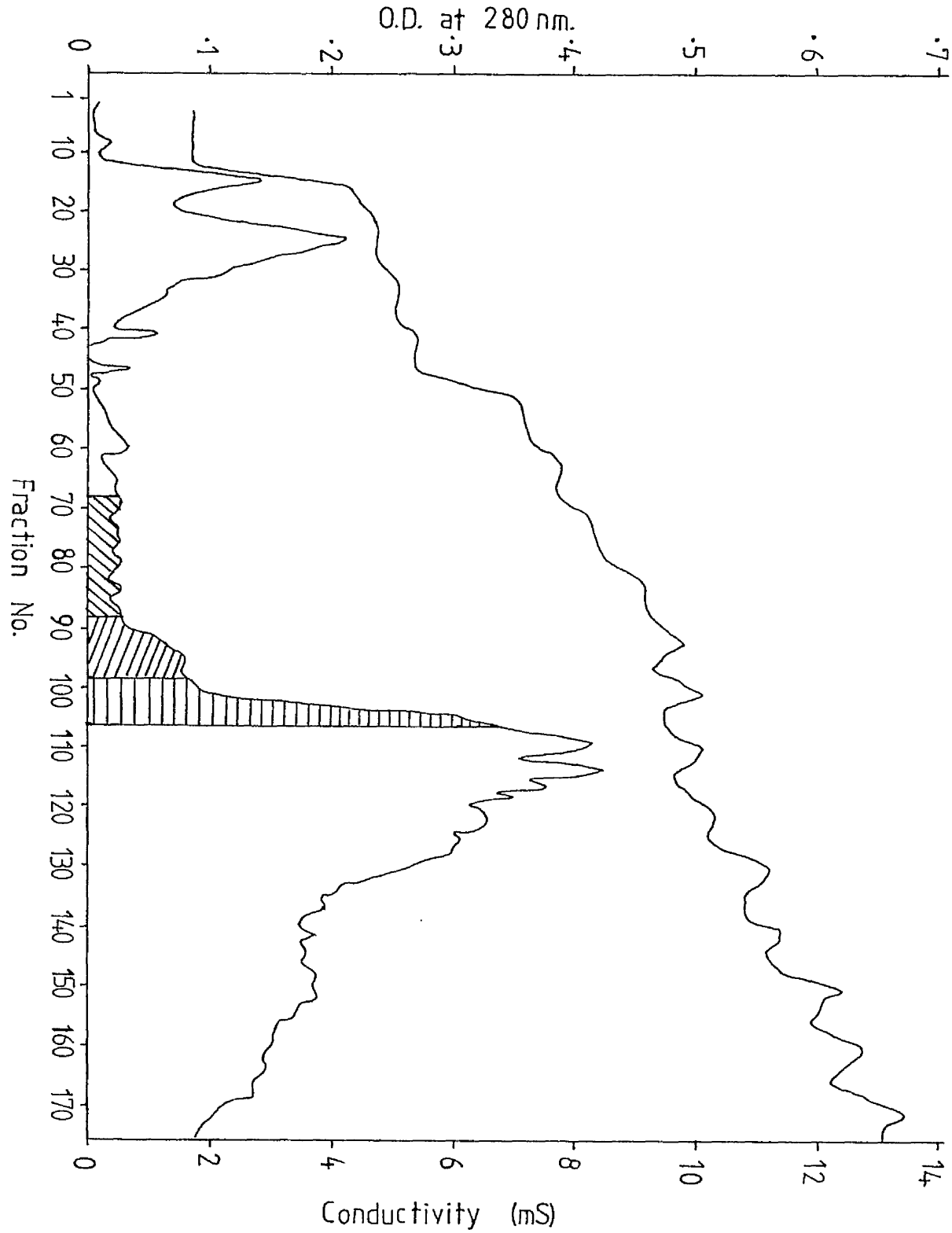

O.D. at 280 nm.
0
·1
·2
·3
·4
·5
·6
·7
Fraction No.
1
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
0
2
4
6
8
10
12
14
Conductivity (mS)

Fig. 23.

Table 7. Protein and AUP concentrations of the pooled ion exchange material.

	Pool	Fractions pooled (nos.)	Volume (ml)	Protein conc (mg/ml)	Protein (mg)	AUP conc (units/ml)	AUP (units)	AUP/protein (units/mg)
Run 1	1	68-87	11.4	0.44	5.02	0.64	7.30	1.45
	2	88-97	12.2	0.27	3.29	2.32	28.30	8.60
	3	98-105	9.5	1.39	13.21	5.18	49.21	3.73
Run 2	1	65-70	9.1	0.67	6.10	NT	NT	NT
	2	71-93	12.4	0.62	7.69	0.40	4.96	0.64
	3	94-109	22.8	5.68	129.50	0.49	11.17	0.09

NT = not tested

Table 8. Enrichment of AUP after Con-A Sepharose chromatography of crude amniotic fluid.

	Volume (ml)	Conc of AUP (units/ml)	AUP (units)	Protein (mg)	AUP/protein (units/mg)
Material applied to column	10	1.86	18.6	53.30	0.35
Material eluted from column	9	0.59	5.31	4.41	1.20

Table 9. Recovery of AUP after Con-A Sepharose chromatography of pooled ion exchange eluate fractions.

Material	Vol (ml)	AUP (units/ml)	AUP (units) X	Protein (mg) Y	AUP/protein (units/mg) X/Y
Applied to column	28	1.85	51.80	21.84	2.37
Not retained by column	39.5	-	-	15.28	-
∴ Retained before elution	-	-	51.80	6.56	7.90
Eluted in fractions 62-68	8	1.33	10.64	2.64	4.03
% recovery			20.5		

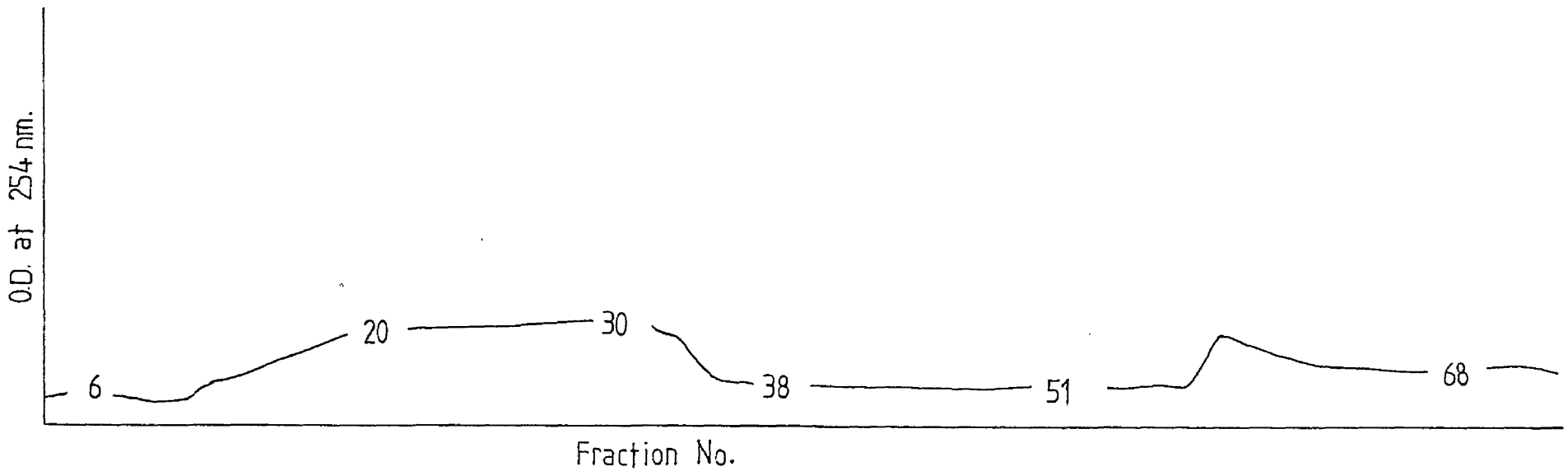


Fig. 24. Spectrophotometric trace (absorbance at O.D.254) of protein dissociated during Con-A Sepharose chromatography of pooled ion exchange eluates.

Fig. 25.

a) One-dimensional AACE illustrating the serum protein contaminants present in run through fractions from Con-A Sepharose chromatography of partially-purified AUP. Antiserum: 4% S_1S_2, a sheep antiserum to adult human serum. Antigens (L - R): Amniotic fluid (control) (5 µl), fraction nos. 30-43 from Con-A Sepharose column (10 µl of each fraction).

b) One-dimensional AACE of the serum protein contaminants in fractions eluted from the Con-A Sepharose column. Antiserum: 4% S_1S_2. Antigens (L - R): Amniotic fluid (control) (5 µl), fraction nos. 57-65 (10 µl of each fraction).

Fig. 25a)

Fig. 25b)

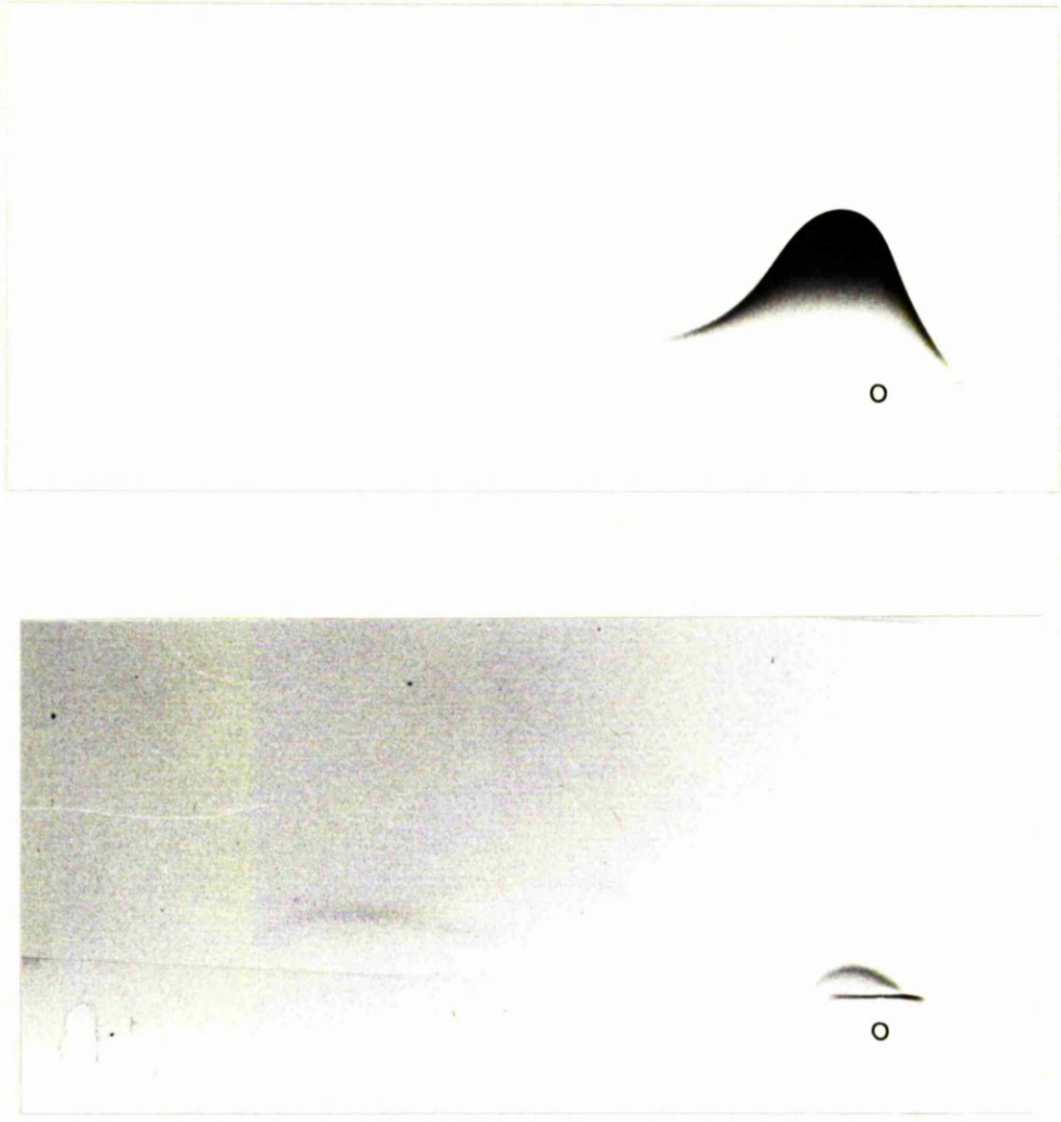


Fig. 26. Semi-quantitative assay by two-dimensional AACE of the residual serum albumin present after Con-A Sepharose chromatography of AUP partially-purified by ion exchange chromatography. Antiserum (both plates) : 4% S_1S_2.

Antigens: Upper plate. Pooled ion exchange fractions 68-87 + 98-105 + 71-93 (4.3 µl).

Lower plate. Pooled fractions 62-68 from Con-A Sepharose column (6 µl).

Both antigens contained approximately 8 µ units of AUP. The single circle at the RHS of each plate denotes albumin mobility in the first dimension.

7. Radioiodination and analysis of partially-purified AUP

Introduction: In the preceding section partial purification of AUP from human amniotic fluid was reported. In order to determine the nature and degree of purity of this material, an aliquot was radiolabelled with ^{125}Iodine and the ^{125}I-labelled AUP analysed by SDS-polyacrylamide gel electrophoresis after extraction from a precipitin arc on AACE.

Methods: A total of four 10 µl aliquots of partially-purified AUP (fractions 62-68 eluted from the Con-A Sepharose column) were radioiodinated by the chloramine-T method of Hunter and Greenwood (1962). After the iodination reactions, labelled protein was separated from free radioiodide by passage through a small column of Sephadex G-25 (Pharmacia).

One-dimensional AACE: In a preliminary experiment, 5 µl volumes of the radiolabelled material were combined with 5 µl of unlabelled amniotic fluid as a source of carrier AUP, and reacted against each of anti-AUP serum (raised in rabbit against decidual AUP) and sheep antiserum to adult human serum. To control for antiserum specificity, 2 µl of adult human serum was added to each alternate well on the AACE plates. A second AACE experiment was carried out in a similar manner. This time, 5 µl volumes of cold amniotic fluid carrier were combined with varying amounts of the tracer from 5-20 µl. AUP was assayed using two different antisera, raised against decidual and amniotic fluid AUP, respectively. As an additional control for nonspecific binding of the tracer, another AACE plate was run using serum from a non-immunised rabbit instead of a specific antiserum. All the AACE plates were dried

and the resultant precipitin arcs detected by autoradiography using Kodak NS-2T film.

SDS-polyacrylamide gel electrophoresis: After autoradiography, the AACE plates were stained with 2% Coomassie blue and the AUP precipitin arcs cut out using a scalpel. These were placed in protease-free vials (3-4 arcs per vial) and incubated in β-mercaptoethanol at 100°C for 2 hours to extract the protein then applied to a 4.5-12.5% SDS-polyacrylamide slab gel. Carbonic anhydrase (MW 29,182) and pyruvate dehydrogenase were run alongside as molecular weight standards. The pyruvate dehydrogenase (PDH) was extracted from E. coli, and consists of three chains of MW 100,000, 80,000 and 56,000, respectively (Coggins et al, 1976). The slab gel was dried and stained with 0.2% Coomassie blue then subjected to autoradiography as before. Protein mobilities were estimated relative to the distance between the top of the running gel and the marker dye front (Bromophenol blue, BDH indicators), which was said to have an Rf value of 1. (see main "Materials and Methods" section for further details of SDS-polyacrylamide gel electrophoresis).

Results: After radioiodination the specific activities of the four aliquots of partially-purified AUP were 83, 79.25, 43.3 and 21 μCi/ml, respectively. In the preliminary AACE experiment the tracer of highest specific activity was used. The autoradiograph of the two AACE plates (AUP and serum protein assays) is shown in Fig 27. In the AUP assay most of the radioactivity was concentrated inside the precipitin arc rather than on the periphery. In the serum protein assay radioactivity was concentrated at the tip of the major precipitin arc which probably represents serum albumin. The addition of adult serum caused this major precipitin arc to "rocket". In the second AACE

experiment radiolabelled aliquots of specific activities 79.25 and 43.3 μCi/ml were used. Four AACE plates were run, namely two AUP assays, a serum protein assay and a control for non-specific binding of the tracer. The resultant precipitin arcs were again examined by autoradiography. The number of serum proteins present in the tracer were indistinct on the autoradiograph but about 8 components were visible after staining of the AACE plate. Again, addition of adult serum to the tracer resulted in "rocketing" of the major precipitin peak. Background radiation was detected on the specificity control plate indicating that there was some non-specific binding of the tracer to the gel. In the two AUP assays performed using antisera raised against decidual and amniotic fluid AUP, respectively, the addition of increasing volumes of tracer to a constant volume of cold carrier AUP resulted in a progressive increase in the height of the resultant precipitin arcs. This is additional evidence of the specificity of ^{125}I-AUP. In both the AUP assays, radioactivity was again concentrated within the precipitin peaks. (see Fig 28).

SDS-polyacrylamide gel electrophoresis: A total of 5 samples of precipitated ^{125}I-AUP extracted from AACE were applied to a slab gel as described in the Materials and Methods section. On staining the gel, however, no bands other than those of the molecular weight standards were visible. However, three bands were detected by autoradiography of the gel in each of the 5 samples of ^{125}I-AUP analysed (see Fig 29). The molecular weights of these three bands were estimated by comparing their mobilities with those of the molecular weight markers as shown in Fig 30. The three components present in the ^{125}I-AUP were found to have molecular weights of 48,000, 16,000 and 9,000 daltons, respectively.

Discussion: The above results indicate that AUP was successfully radioiodinated and that the antigenicity of this protein was unaltered by the radioisotope, ie. no apparent denaturation of AUP occurred. In the AACE autoradiographs, the discovery of a concentration of radioactivity within the AUP precipitin peaks may correlate with the previous observation that AUP reacted against antiserum to decidual AUP resulted in the production of "filled-in" peaks on AACE (see section 2 of "Results"). A more likely hypothesis is that this reflects non-specific binding of the tracer to the gel, since there is evidence that this occurred. In addition, in the second AACE experiment the counts were concentrated inside the AUP peaks when the antiserum used was raised against either decidual or amniotic fluid AUP.

It was also apparent from the AACE experiments that the partially-purified AUP contains several serum protein contaminants. An attempt was made to isolate AUP from these contaminants by extracting ^{125}I-AUP from AUP precipitin arcs on AACE. This material contained three components: a major band of MW 48,000 and two minor bands of 16,000 and 9,000 molecular weight, respectively. It is possible that the major band represents non-denatured AUP in view of the similarity between the MW of 48,000 and that estimated for AUP by gel filtration on Sephadex G-150. In a previous report evidence was presented that AUP is a dimer composed of two similar or identical subunits of approximately 25000 molecular weight (Sutcliffe et al, 1980). However, from the results of this study it appears possible that these subunits may undergo further degradation, to yield two peptide chains of 16000 and 9000 molecular weight respectively. Thus, AUP may be a tetramer composed of two peptide chains of MW 16000 and two peptide chains of MW 9000. Alternatively, the two minor bands visualised on the SDS-polyacrylamide

gel may represent low MW serum proteins or subunits of serum proteins which were present as contaminants in the partially-purified AUP. It is unlikely that serum proteins would have been immune precipitated on AACE using anti-AUP serum because the antiserum was adsorbed of serum specificities. However, due to the fact that some non-specific binding of the tracer to the agarose gel on AACE was observed, it is possible that some serum proteins were extracted from the gel along with the precipitated AUP.

Clearly, further physicochemical studies using pure AUP are required to resolve the question of the subunit structure of this protein.

Fig. 27. Upper: Autoradiograph of one-dimensional AACE of ^{125}I-labelled AUP. Each well contained 5 µl ^{125}I-AUP (partially-purified) and 5 µl amniotic fluid (cold carrier AUP). Wells 2 and 4 (numbering from L - R) also contained 2 µl of adult human serum as a specificity control. The antiserum was raised in rabbit against decidual AUP and used at a final concentration of 20%.

Lower: Autoradiograph of one-dimensional AACE of the serum proteins present in ^{125}I-labelled partially-purified AUP. Contents of the wells are as described above. The antiserum was 4% sheep antiserum raised against adult human serum.

Fig. 28. Autoradiograph of one-dimensional AACE of ^{125}I-labelled AUP. The four wells in each case contain 5 µl cold carrier AUP combined with (from L - R) 5,10,12 and 15 µl ^{125}I-AUP (partially-purified). The antisera were as follows:

Upper plate: Antiserum raised in rabbit against decidual AUP, used at a final concentration of 20%.

Lower plate: Rabbit antiserum to amniotic fluid AUP, used at a final concentration of 20%. Both antisera were adsorbed with adult human serum.

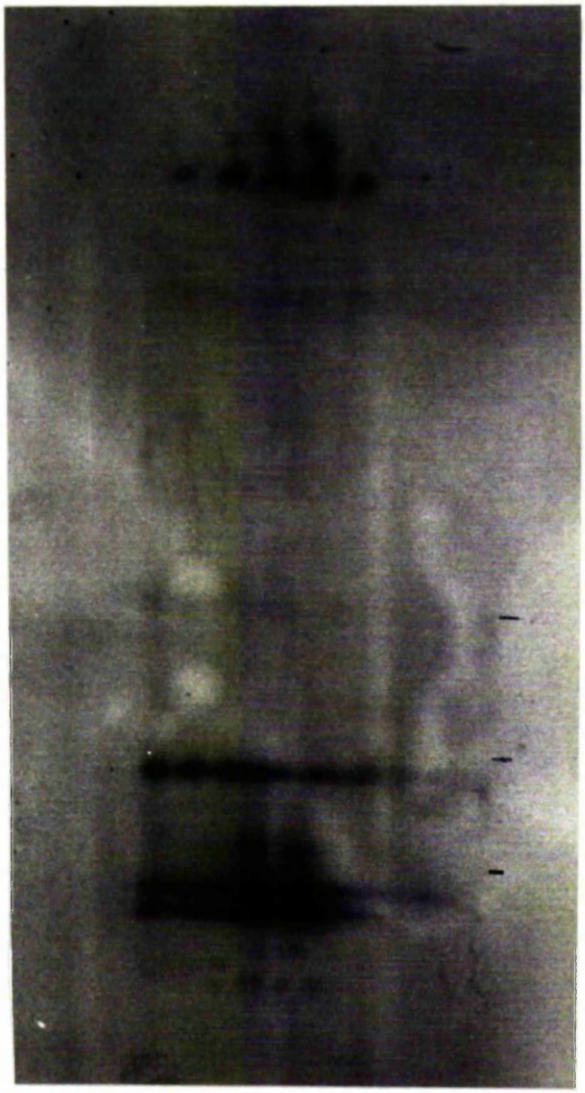


Fig. 29. Autoradiograph of the five separate samples of ^{125}I-AUP extracted from AUP precipitin arcs and analysed by SDS-polyacrylamide gel electrophoresis under denaturing conditions. The small lines at the RHS of the photograph indicate the positions of the MW standards. These are (from top to bottom): 100,000 MW component of PDH, 80,000 MW component of PDH, 56,000 MW component of PDH and carbonic anhydrase. The three bands visible on the autoradiograph correspond to ^{125}I-labelled components of molecular weights (from top to bottom) 48,000, 16,000 and 9,000.

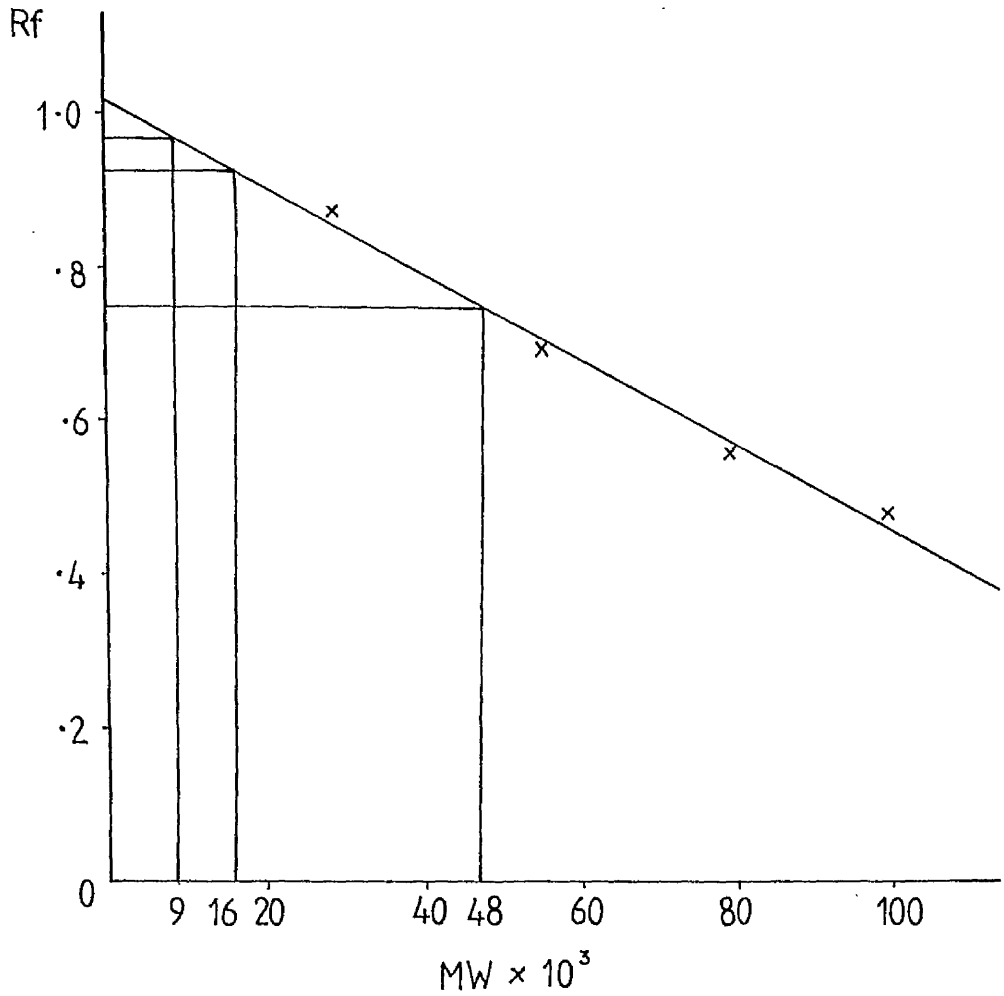


Fig. 30. Estimation of the molecular weight of the subunits of ^{125}I-labelled AUP by SDS-polyacrylamide gel electrophoresis. The mobility of the AUP components were compared to the standard curve shown above. Carbonic anhydrase and PDH were used as molecular weight standards to construct the curve.

8. Measurement of human serum and non-serum proteins in second trimester amniotic fluid

Introduction: Alpha uterine protein is present in amniotic fluid and the concentration changes of this protein from 10 weeks of gestation to term have been measured (Sutcliffe et al, 1978). However the origin of AUP in amniotic fluid is not known. The similarity between the concentration profile of AUP in amniotic fluid during the second and third trimesters with that of two other non-serum proteins, α1,4-glucosidase and prolactin, suggests that these three proteins may have a common origin during this period. The consistent presence of AUP in both pregnant and nonpregnant uterine tissue indicates that the uterus may be the major site of synthesis of this protein, hence the tissue of origin of amniotic fluid AUP. In addition, there is evidence that amniotic fluid prolactin originates from uterine decidua and possibly chorion (Riddick and Kusmik, 1977; Golander et al, 1978). The origin of α1,4-glucosidase in amniotic fluid is uncertain, although a maternal or fetal nonserum source appears to be likely.

In contrast to the above-mentioned proteins, the quantitatively major proteins in amniotic fluid, which are of serum origin, attain maximal concentrations between 20 and 30 weeks of gestation (Sutcliffe and Brock, 1973). Evidence from genetic and quantitative studies indicates that these proteins in amniotic fluid have a maternal rather than fetal origin (Abbas and Tovey, 1960; Strebel, 1960; Derrington and Soothill, 1961; Ruoslahti et al, 1966; Seppälä et al, 1966; Usategui-Gomez and Morgan, 1966; Gitlin and Biasucci, 1969; Sutcliffe et al, 1972; Johnson et al, 1974; Sutcliffe, 1975). However there is some

conflicting evidence for albumin (Brzezinski et al, 1964) and α_1-antitrypsin (Kaiser et al, 1974).

In this study, the quantitative relationships between AUP, prolactin and α1,4-glucosidase in amniotic fluid are investigated by measuring the concentrations of these proteins in amniotic fluid between 16 and 19 weeks of gestation. Clearly, any correlation between these three proteins could argue in support of a common origin in amniotic fluid. The concentrations of some serum proteins in amniotic fluid were also measured in order to further examine the origins of albumin and α_1-antitrypsin.

Methods: Twenty human amniotic fluid samples ranging from 16 to 19 weeks of gestation were obtained by amniocentesis. All pregnancies proceeded normally and resulted in the delivery of normal infants. After centrifugation at 10,000 g for 15 mins, the fluids were stored at -20°C prior to use. The concentrations of the seven proteins were assayed using the following methods. Alpha uterine protein, α_1-antitrypsin, group-specific component and albumin were assayed by one-dimensional antibody-antigen crossed electrophoresis on 1% agarose gel as described by Brock and Sutcliffe (1972). The concentration of AUP was expressed arbitrarily as "units per ml sample", relative to a reference amniotic fluid sample. The concentration of α_1-antitrypsin, Gc and albumin were calculated relative to reference standards of known concentration. The sources of these standards were "Protein-Standard-Serum Lc-V" and "Standard-Human-Serum, stabilized", both produced by Behringwerke AG, West Germany. AUP was assayed using an antiserum described previously (Sutcliffe et al, 1979). Commercial antisera (Behringwerke), raised in rabbit were used to assay α_1-antitrypsin, Gc-globulin and albumin. These antisera were used at a final concen-

tration of 2%. Alpha 1,4-glucosidase was assayed by the method of Sutcliffe et al (1972). Alpha fetoprotein was assayed by the method of Allan et al (1973). The concentration of prolactin was measured by radioimmunoassay. The prolactin assay was kindly carried out by Dr. A.M. Wallace, Senior Biochemist, Dept. of Biochemistry, Royal Infirmary, Glasgow. A statistical analysis of the results of the protein assays was carried out by computer, using the "Minitab" programme (Ryan et al, 1976).

Results: The concentrations of the various proteins were measured in 20 independent samples of cell-free amniotic fluid and the mean concentrations and their ranges are shown in Table 10. The prolactin values recorded represent the mean of three assays performed at sample dilutions of 1:100, 1:500 and 1:1000. Due to a non-linear response at different dilutions in the majority of the samples the prolactin results were not included in the statistical analysis. Table 11 summarises the correlation coefficients for each pair of proteins. Because of the multiple correlations to be carried out it was not necessarily admissible to accept a correlation as significant at $p \leq 0.05$ since, on random sampling one might expect 5% of correlations to be positive. The correlation matrix was therefore divided into two, placing the serum proteins (Gc, AFP, albumin and α_1-antitrypsin) into one matrix and the nonserum proteins into another. The modified values of r and p for hypothesis testing are shown in Table 11. Three positive correlations were found, between albumin and α_1-antitrypsin ($r = 0.594$), albumin and Gc ($r = 0.647$) and between Gc and α_1-antitrypsin ($r = 0.814$). No correlation was found between AUP and α1,4-glucosidase ($r = 0.024$). These results are shown graphically in Fig 31 a) - d).

Discussion: The concentrations of α_1-antitrypsin, albumin, α1,4-glucosidase, AUP and AFP are similar to levels previously reported at this stage of gestation (Sutcliffe et al, 1972b, 1978; Sutcliffe and Brock, 1973; Weiss et al, 1978). In the amniotic fluids assayed, the Gc concentration ranged from 12-34 mg/L, with a mean value of 24.2 mg/L. There have been no other studies on Gc levels in amniotic fluid at this stage of gestation. However, Brock and Nelson (1974) reported a mean Gc concentration of 32 mg/L in amniotic fluid between 29-42 weeks of gestation. Taking into account the pattern of concentration changes of Gc throughout gestation, these two mean values appear to be compatible.

From the table of correlation coefficients, it is clear that there is no quantitative relationship between α1,4-glucosidase and AUP in the amniotic fluid samples examined. Thus the origin of the α1,4-glucosidase in the amniotic fluid remains undecided. In the prolactin assay a non-linear response was observed at different dilutions. To my knowledge there have been no reports of a similar response in assays of human pituitary prolactin. This may suggest that the molecular form of amniotic fluid prolactin differs from that of human pituitary prolactin. This appears unlikely since there is evidence that amniotic fluid prolactin is identical to human serum and pituitary prolactin as regards molecular size, net charge, biological activity and immunoactivity (Ben-David et al, 1973; Ben-David and Chrambach, 1977). However, it has been reported that storage of prolactin for several months at -20°C results in a reduction in size of the hormone from a molecular weight of 30,000 to approximately 20,000 daltons (Rogol and Chrambach, 1975). This may reflect either proteolysis or re-folding of the molecule in the frozen state. Since the amniotic fluid samples used in this study were previously stored at -20°C the

results observed may reflect such an alteration of the prolactin molecule.

In contrast to the lack of correlation between the non-serum proteins in amniotic fluid, positive correlations are evident between the three serum proteins: albumin, Gc and α_1-antitrypsin (Table 11, Fig 31 a)-c)). Since the Gc in amniotic fluid is known to be mainly of maternal serum origin (see main "Introduction"), these correlations provide further evidence that the albumin and α_1-antitrypsin in second trimester amniotic fluid are also mainly of maternal serum origin.

Table 10. The mean and range of proteins in cell-free amniotic fluid

Gestation weeks	No. of samples		Albumin mg/ml	Gc mg/L	α_1-anti-trypsin mg/L	AFP mg/L	AUP units/ml	α1,4-glucosidase μmole/μl/hr.	prolactin mU/L x 10^2
16	6	mean	3.06	20	98	15.2	1.59	1.07	218
		range	(1.75-4.56)	(13-29)	(30-160)	(13.0-17.9)	(0.46-2.96)	(0.61-1.68)	(101-415)
17	9	mean	3.92	25	154	13.4	1.33	0.72	308
		range	(2.54-7.26)	(12-34)	(60-250)	(4.9-22.4)	(0.31-4.11)	(0.44-1.15)	(225-575
18-19	5	mean	3.96	27	180	11.5	1.81	0.69	338
		range	(3.29-5.57)	(22-32)	(120-230)	(1.0-16.4)	(0.37-3.74)	(0.48-0.99)	(191-550)

Table 11. Correlation Coefficients (r) for serum and non-serum proteins in amniotic fluid.

	Albumin	Gc	α_1-antitrypsin
Gc	0.647*		
α_1-antitrypsin	0.594*	0.814*	6 comparisons
AFP	-0.565	-0.054	-0.195

	AUP	
α-1,4-glucosidase	0.024	1 comparison

*Significant correlation. The critical value, p^n, the probability of rejecting the null hypothesis in any one of n comparisons, was calculated as p/n, where n = number of comparisons in the matrix and p was set at 0.05.

for n = 6, p^n = 5/6 = 0.83%, r = 0.58 for 18 df

for n = 1, p^n = 5/1 = 5.0%, r = 0.44 for 18 df

Fig. 31. a) - c). Graphs of the positive correlations between levels of serum proteins in 20 separate samples of 2nd trimester amniotic fluid.

a) Gc and albumin

b) α_1-antitrypsin and albumin

c) α_1-antitrypsin and Gc

d) Graph illustrating the lack of correlation between levels of AUP and α1,4-glucosidase in 20 separate samples of 2nd trimester amniotic fluid.

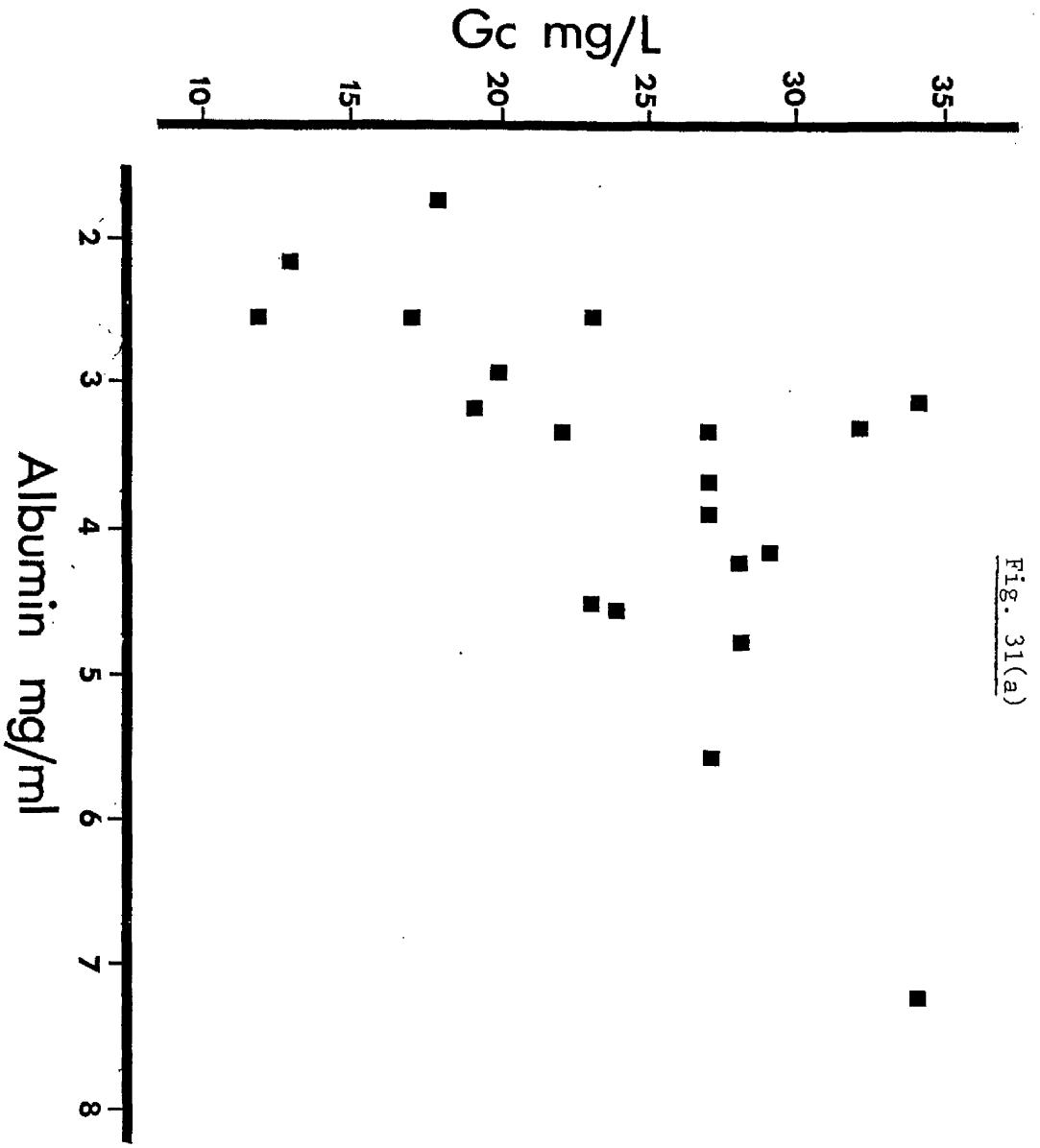


Fig. 31(a)

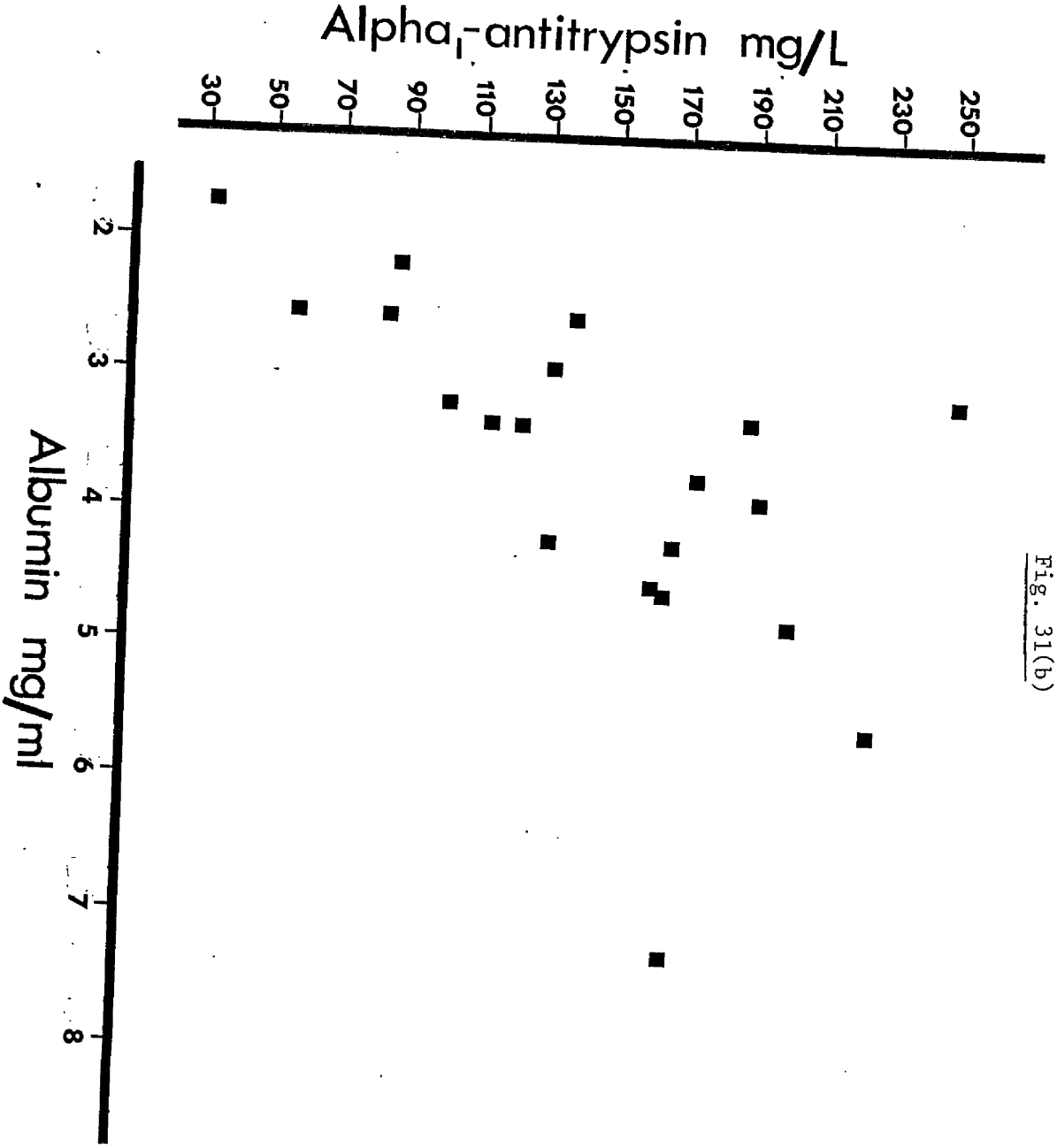

Alpha$_1$-antitrypsin mg/L
30
50
70
90
110
130
150
170
190
210
230
250
Albumin mg/ml
2
3
4
5
6
7
8

Fig. 31(b)

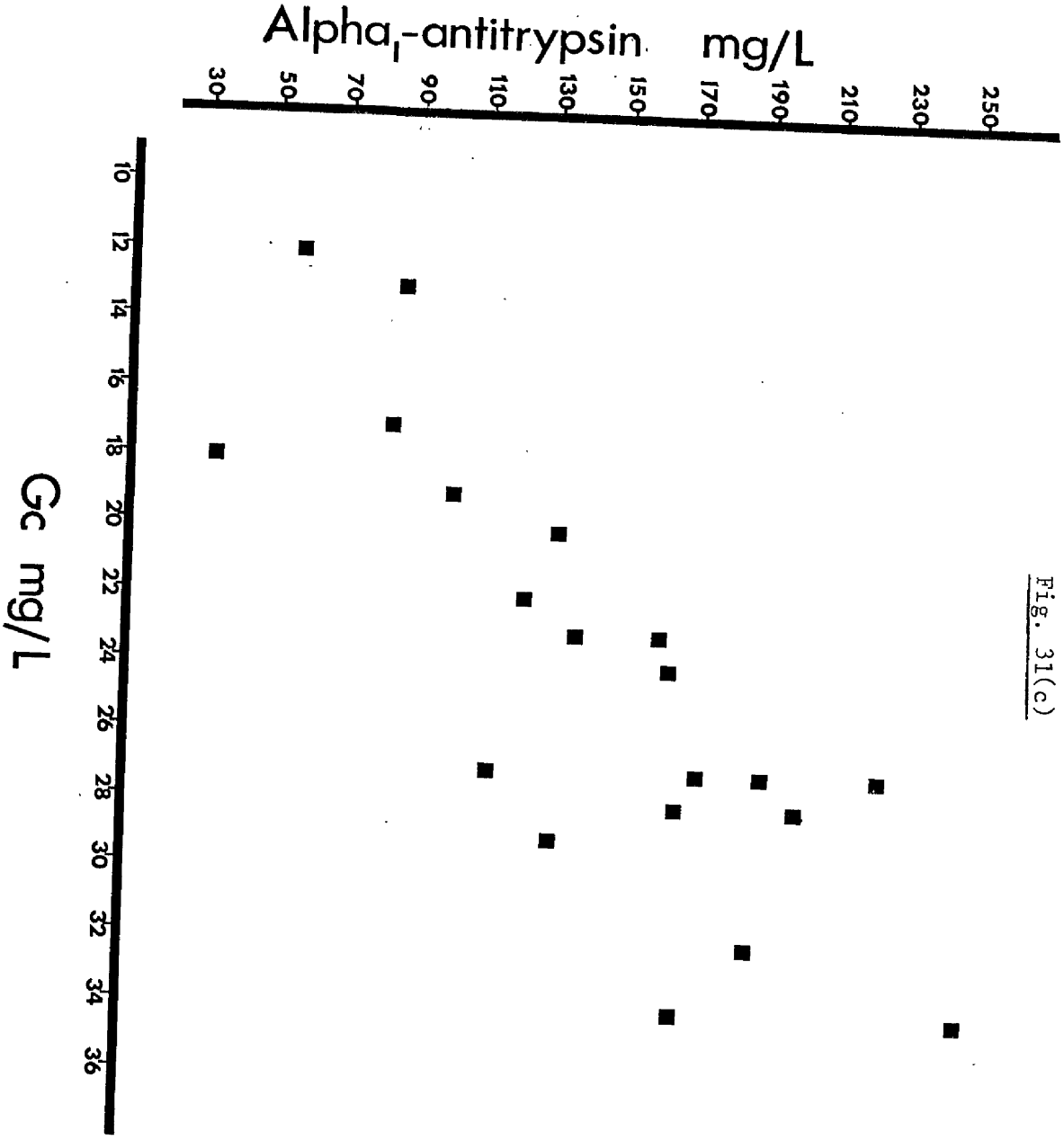

Alpha$_1$-antitrypsin mg/L
30
50
70
90
110
130
150
170
190
210
230
250
Gc mg/L
10
12
14
16
18
20
22
24
26
28
30
32
34
36

Fig. 31(c)

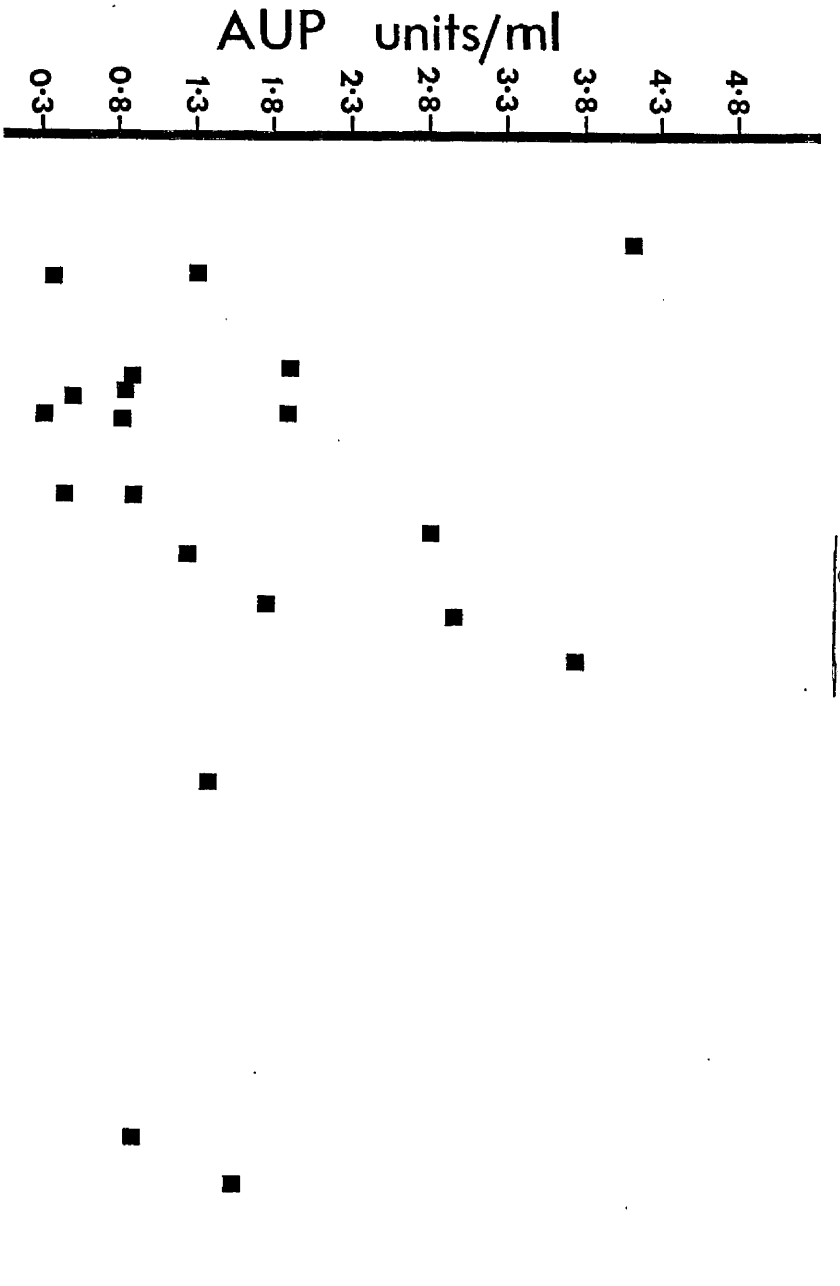

AUP units/ml
0·3
0·8
1·3
1·8
2·3
2·8
3·3
3·8
4·3
4·8
α 1,4-glucosidase μmoles/ml/hr
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·1
1·2
1·3
1·4
1·5
1·6
1·7

Fig. 31(d)

9. An immunological comparison of Alpha uterine protein and Progestagen-dependent endometrial protein

Introduction: Progestagen-dependent endometrial protein (Joshi et al, 1980a,b) and alpha uterine protein are similar as regards glycoprotein nature, molecular size and subunit structure. In addition, both proteins are believed to be uterine-specific. Therefore, it is possible that AUP and PEP are the same protein. To investigate this hypothesis, anti-AUP serum and anti-PEP serum were tested for possible immunological cross-reactivity on a double immunodiffusion system.

Methods: Ouchterlony double immunodiffusion was carried out as described in the main "Materials and Methods" section. Anti-AUP serum was raised in rabbit against partially-purified AUP from 11 week gestation decidua (Sutcliffe et al, 1980) and adsorbed with adult human serum. Rabbit anti-PEP serum was raised against partially-purified PEP and adsorbed with adult human serum. A glycoprotein fraction of decidua was used as a source of PEP. The latter two reagents were kindly provided by Prof. S.G. Joshi, Dept. of Obstetrics and Gynecology, the Neil Hellman Medical Research Building, Albany Medical College, Albany, New York 12208, U.S.A.

Results: A reaction of complete identity was observed between anti-AUP and anti-PEP on double immunodiffusion. This is illustrated in Fig 32 and 33. This result was confirmed in the New York laboratory.

Discussion: The results of this experiment indicate that AUP and PEP are the same protein. From studies carried out on this protein to date (Sutcliffe et al, 1978, 1979,1980; Joshi et al, 1980a,b) a considerable amount of information is available, particularly regarding

its physicochemical properties. However, as previously discussed (see section 4 of "Results") the complete distribution of this protein is not yet known. Alpha uterine protein has been detected by one-dimensional AACE in human endometrium, myometrium, decidua, amnion, chorion, amniotic fluid, early gestation (8-13 weeks) and term placentae, and fetal gut (Sutcliffe et al 1978, 1979 + unpublished observations). In contrast, the presence of PEP has only been reported in human endometrium and decidua; it could not be detected in the other tissue examined so far, namely term placenta (Joshi et al, 1980a). This discrepancy may reflect the fact that Joshi and colleagues used the techniques of double immunodiffusion and immunoelectrophoresis which are less sensitive than AACE to detect PEP.

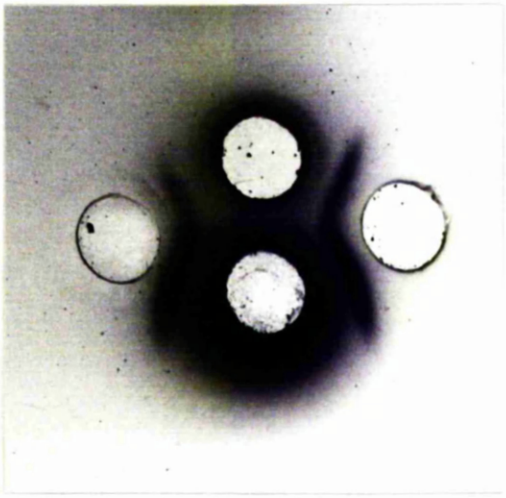


Fig. 32. Ouchterlony double immunodiffusion experiment illustrating immunological identity of alpha uterine protein (AUP) and progestagen-dependent endometrial protein (PEP). The following antigens and antisera were introduced into 4 mm diameter wells cut in 1% agarose. (Starting at the top and reading clockwise):

Well 1 : anti-PEP serum (10 µl).

Well 2 : pooled human amniotic fluid (54.8 µg protein; 10 mU AUP).

Well 3 : anti-AUP serum (20 µl).

Well 4 : decidual glycoprotein fraction (source of PEP). (10 µl).

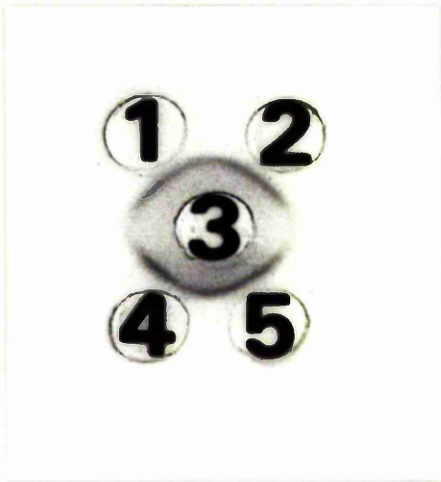


Fig. 33. Ouchterlony double immunodiffusion experiment illustrating serological identity of anti-AUP serum and anti-PEP serum. Volumes (10 μl) of the following antigens and antisera were introduced into 4 mm diameter wells cut in 1% agarose.

Wells 1 and 5 : pooled human amniotic fluid (54.8 μg protein; 10 mU AUP).

Wells 2 and 4 : decidual glycoprotein fraction (source of PEP).

Well 3 : anti-PEP serum (5 μl) plus anti-AUP serum (5 μl).

10. Progesterone-binding studies

Introduction: The physiological function of alpha uterine protein is not yet known. However, the presence of this protein in the uterine decidua, placenta, fetal membranes and amniotic fluid suggests that it has a pregnancy-associated function. One possibility is that AUP binds the steroid hormone progesterone. Progesterone is produced throughout gestation by the placenta, metabolised in fetal tissues then returned to the placenta for further conversion. Therefore progesterone receptor(s) may be involved in the transportation of this steroid hormone. A progesterone-receptor protein of 54000 molecular weight has been reported in the cytosol of human endometrium and myometrium (Janne et al, 1975). The isoelectric point of this receptor was 4.8 with a second minor peak focusing at pH 5.2. A similar isoelectric point of about 4.9 was reported for progestagen-dependent endometrial protein (Joshi et al, 1980b). Therefore it is possible that AUP/PEP may represent the uterine progesterone-receptor described by Janne et al (1975).

Methods: (1,2-^{3}H)Progesterone of specific activity 40-60 Ci/mmole was obtained from The Radiochemical Centre, Amersham. The source of AUP was material partially-purified from amniotic fluid by ion exchange chromatography (the first stage of the two-step purification procedure described in "Results" section 6). This partially-purified material contained 0.6 mg/ml protein and was enriched with AUP, having a concentration of approximately 3 units AUP/mg protein compared with approximately 0.2 units AUP/mg protein in the original amniotic fluid, ie. the AUP concentration was increased 15-fold after ion exchange chromatography. Progesterone-binding was measured by equilibrium dialysis using a method adapted from those described by Fridlansky and Milgrom (1976)

and Pollow et al (1978). A preliminary experiment was carried out in order to find the time required to reach equilibrium. The AUP solution (1 ml) was dialysed (2-8 x 32" dialysis tubing, Medicell International Ltd.) against 15 ml of 20 nmolar (1,2-^{3}H)Progesterone solution containing a 100-molar excess of cortisol at 4^{o}C, with shaking. All the steroid solutions used in the equilibrium dialyses were prepared using 73 nM sodium phosphate buffer, pH 7.4. Cortisol was added to eliminate binding of progesterone by contaminating corticosteroid-binding globulin (transcortin) which specifically binds cortisol, corticosterone, progesterone and 17α-hydroxyprogesterone. At various times from 0-38 hours, a 100 µl aliquot was removed from the solution outside the dialysis bag and the level of radioactivity present counted. In the subsequent equilibrium dialysis experiment 1 ml volumes of partially-purified AUP were dialysed as described above against increasing concentrations of (1,2-^{3}H)Progesterone (0.2, 0.5, 1, 2, 5, 10, 15 and 20 nmolar) in 15 ml of 73 nM sodium phosphate buffer, pH 7.4 at 4^{o}C for the time required to reach equilibrium. As in the preliminary experiment, a constant amount of unlabelled cortisol, 100-fold molar excess over the highest (^{3}H) steroid concentration was added to each steroid solution. As a positive control, an identical equilibrium dialysis experiment was performed using maternal serum of protein concentration 0.6 mg/ml in place of the partially-purified AUP. Maternal serum contains elevated levels of corticosteroid-binding globulin (Klopper and Fuchs, 1977). Therefore in this experiment, cortisol was omitted from the steroid solutions. After dialysis, the vials (16 in total) were removed from the cold room on ice and the contents of the dialysis bags emptied into separate vials. The solutions were allowed to reach room temperature then 100 µl volumes

from each (ie. from inside and outside each dialysis bag) were introduced into separate scintillation vials containing 10 ml Biofluor (New England Nuclear), and counted for 2 minutes in a Liquid Scintillation Spectrometer (Intertechnique, Model SL-30). Each scintillation vial had been precounted before the addition of steroid solution in order to establish the background counts. The results were analysed by Scatchard plot, according to the method of Rosenthal (1967). (see main "Materials and Methods" section for details of Scatchard analysis.)

Results:

Preliminary equilibrium dialysis experiment: As illustrated in Fig 34, the concentration of radioactivity in the solution outside the dialysis bag decreased with time from 40,000 cpm until equilibrium was reached. After the point of equilibrium a constant value of about 32500 cpm was recorded. From these values it was estimated that approximately 0.8% of the total counts added were bound. In the subsequent experiment, dialysis was continued for 30 hours, to ensure that equilibrium was reached.

Equilibrium dialysis: The concentration of radioactivity was measured inside and outside each dialysis bag, and the results shown in Table 12. These data were expressed on Scatchard plots (see Figs 35 and 36). A high affinity progesterone-binding component was evident in the maternal serum control, with an intrinsic association constant (Ka) of 6.56 x $10^{9}M^{-1}$. A considerable increase in the amount of steroid bound was evident at the highest concentration of steroid used (20 nmolar). This may indicate the presence of a nonspecific, low affinity progesterone-binding component of high capacity, ie. the binding sites are only saturated at high concentrations of steroid. In contrast to the maternal serum results, there was no evidence of a correlation between

the bound : unbound ratio and the amount of steroid bound in the case of partially-purified AUP. This suggests a lack of any major specific progesterone-binding component of high affinity. However, as in maternal serum, a marked increase in the amount bound at the highest steroid concentration may indicate the presence of a low affinity component.

Discussion: The specific progesterone-binding component in maternal serum is likely to be corticosteroid-binding globulin (also known as transcortin) which binds cortisol and progesterone with high affinity. The normal range of this receptor protein in adult human female serum is 3.3 ± 0.42 mg/100 ml. During pregnancy, however, the concentration in maternal serum rises, reaching a maximal value of approximately 7 mg/100 ml by the middle of the second trimester, after which the concentration remains stable to term (Doe et al, 1964).

No evidence was found of any specific, high affinity progesterone receptor in the partially-purified AUP. This was illustrated by the lack of correlation on the Scatchard plot. In addition, comparison of the bound to unbound steroid ratio and the amount of steroid bound with the control results reveals that a negligible amount of steroid was bound by partially-purified AUP. However, some binding of progesterone was apparent in the preliminary equilibrium dialysis experiment. This may reflect nonspecific binding by a low affinity ($Ka \leqslant 10^{4} M^{-1}$) receptor, since the preliminary experiment was carried out using the highest steroid concentration (20 nmolar) and the binding sites of low affinity receptors are only saturated at high steroid concentrations. In addition, the AUP solution was partially-purified from amniotic fluid which contains many serum proteins including

albumin and orosomucoid, both of which bind progesterone with low affinity.

Since the concentration of AUP in the partially-purified material is not known, it is possible that AUP does bind progesterone but is not present in a sufficiently high concentration for binding to be detected. Thus it is unlikely for two reasons; firstly specific receptor proteins are characterised by a high association constant ($Ka \geqslant 10^{8}M^{-1}$) ie. steroid is bound even when the receptor is present in very low concentrations (Baulieu et al, 1970). Secondly, AUP is detectable in the partially-purified material by antigen-antibody crossed electrophoresis, therefore lies within the concentration range of 5-50 mg/100 ml (Laurell, 1972). In comparison, the concentration of corticosteroid-binding globulin in maternal serum, which binds progesterone under the same experimental conditions, is at the lower end of this range. Another possibility is that AUP binds progesterone but is an unstable receptor, ie. under the experimental procedure employed either to partially-purify this material or to measure steroid-binding there is a loss of binding activity. Janne et al (1975) reported that the human uterine progesterone receptor was unstable in that attempts to purify it by classical purification techniques resulted in the loss of binding activity. In addition, it was unstable at pH values less than 6.9 and greater than 9. However, the fact that the AUP used in the present experiment was partially-purified by ion exchange chromatography only and equilibrium dialysis was carried out at physiological pH, argues against this possibility.

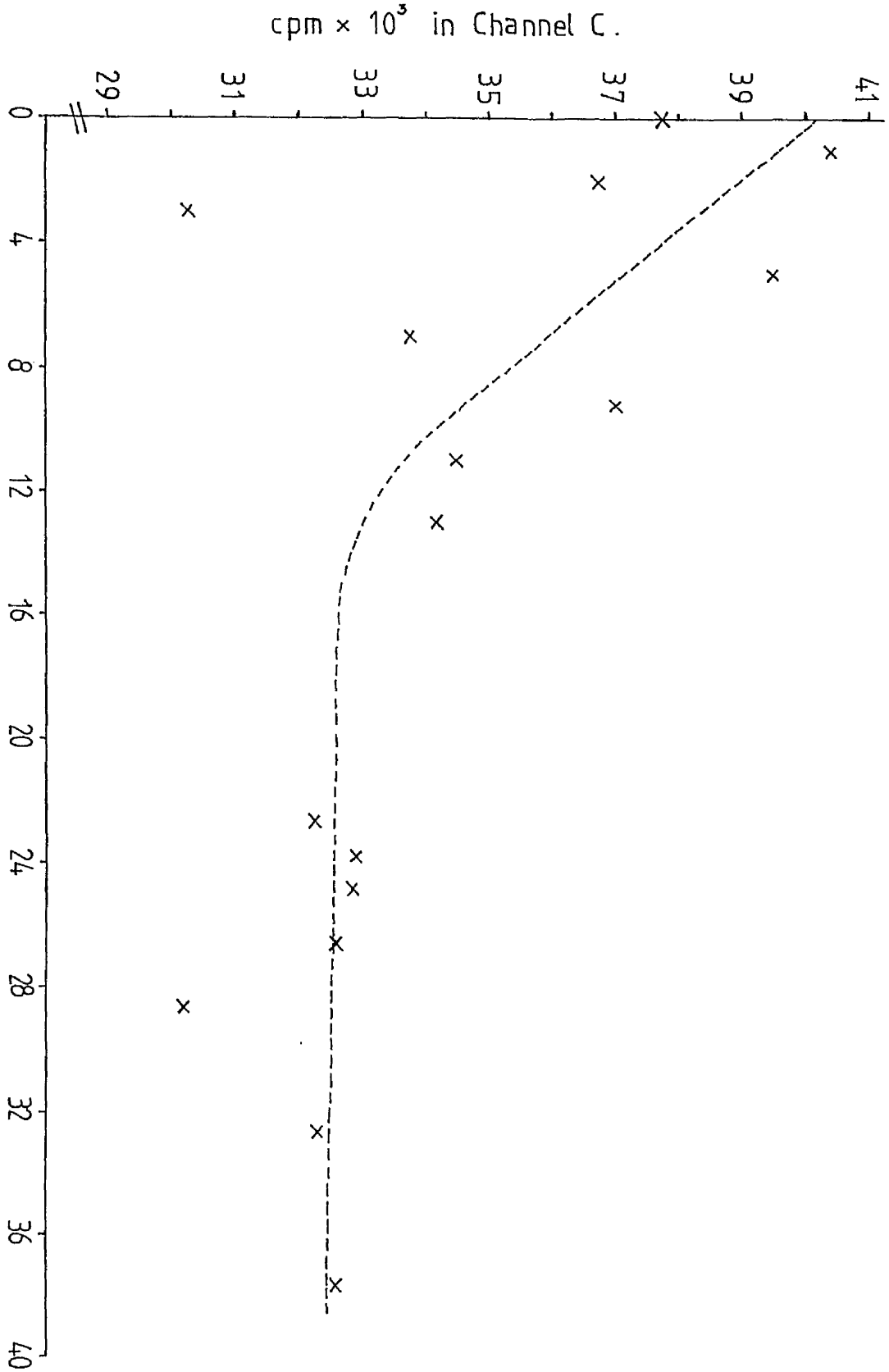


Fig. 34. Preliminary equilibrium dialysis experiment. Kinetics of (1,2-^{3}H) Progesterone binding to partially-purified AUP.

Table 12: Equilibrium dialysis: bound and unbound radioactivity as measured by liquid scintillation counting.

	Conc. of ($1,2\text{-}^3H$) Progesterone (nmolar)	Total steroid added (molesx10^{-9})	Total cpm added (x10^6)	$\frac{(b)}{(u)}$	(b) (moles x 10^{-11})
Partially-purified AUP	0.2	3	0.33	0.026	0.02
	0.5	7.5	0.825	0.058	0.13
	1	15	1.65	0.126	0.69
	2	30	3.3	0.124	1.10
	5	75	8.25	0.056	0.37
	10	150	16.5	0.088	1.60
	15	225	24.75	0.079	1.85
	20	300	33	0.116	10.35
Maternal serum	0.2	3	0.33	6.37	4.61
	0.5	7.5	0.825	5.66	8.39
	1	15	1.65	4.74	16.14
	2	30	3.3	4.95	31.41
	5	75	8.25	4.72	22.48
	10	150	16.5	1.81	71.39
	15	225	24.75	2.72	55.59
	20	300	33	0.56	207.61

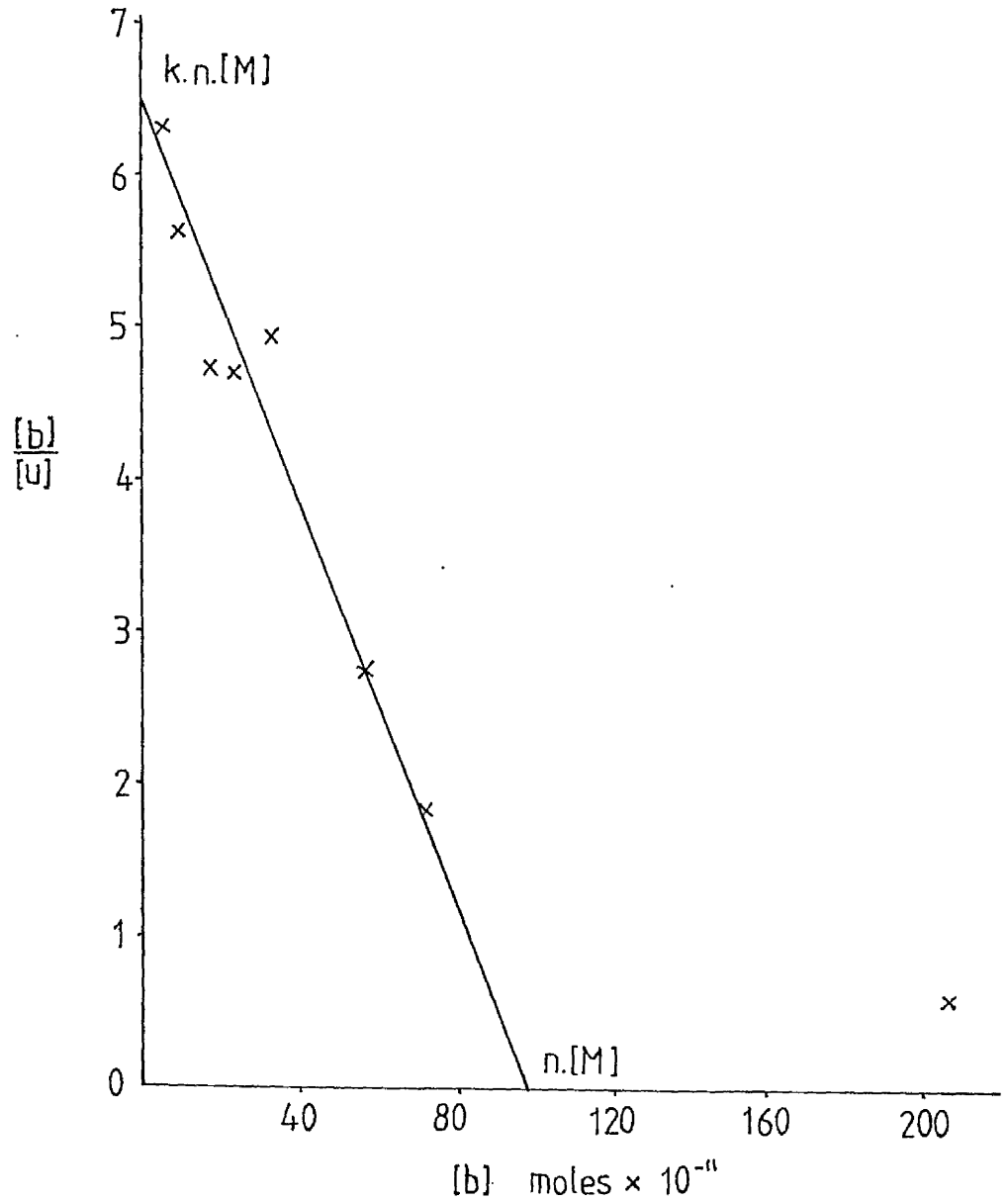


Fig. 35. Scatchard plot of results of progesterone-binding experiment using maternal serum as source of receptor(s). (Control). Slope of line, -k = intrinsic association constant.

k.n.(M) = 6.5 moles steroid bound/0.6 mg protein/ml/mole^{-1}

= 10.83 moles steroid bound/g protein/l/mole^{-1}

n.(M) = 99 x 10^{-11} moles steroid bound/0.6 mg protein/ml

= 165 x 10^{-11} moles steroid bound/g protein/l.

$$\therefore k = \frac{k.n.(M)}{n.(M)} = \frac{10.83}{165 \times 10^{-11}} = \underline{\underline{6.56 \times 10^{9}\ M^{-1}}}$$

(Intrinsic assoc. constant of high affinity component.)

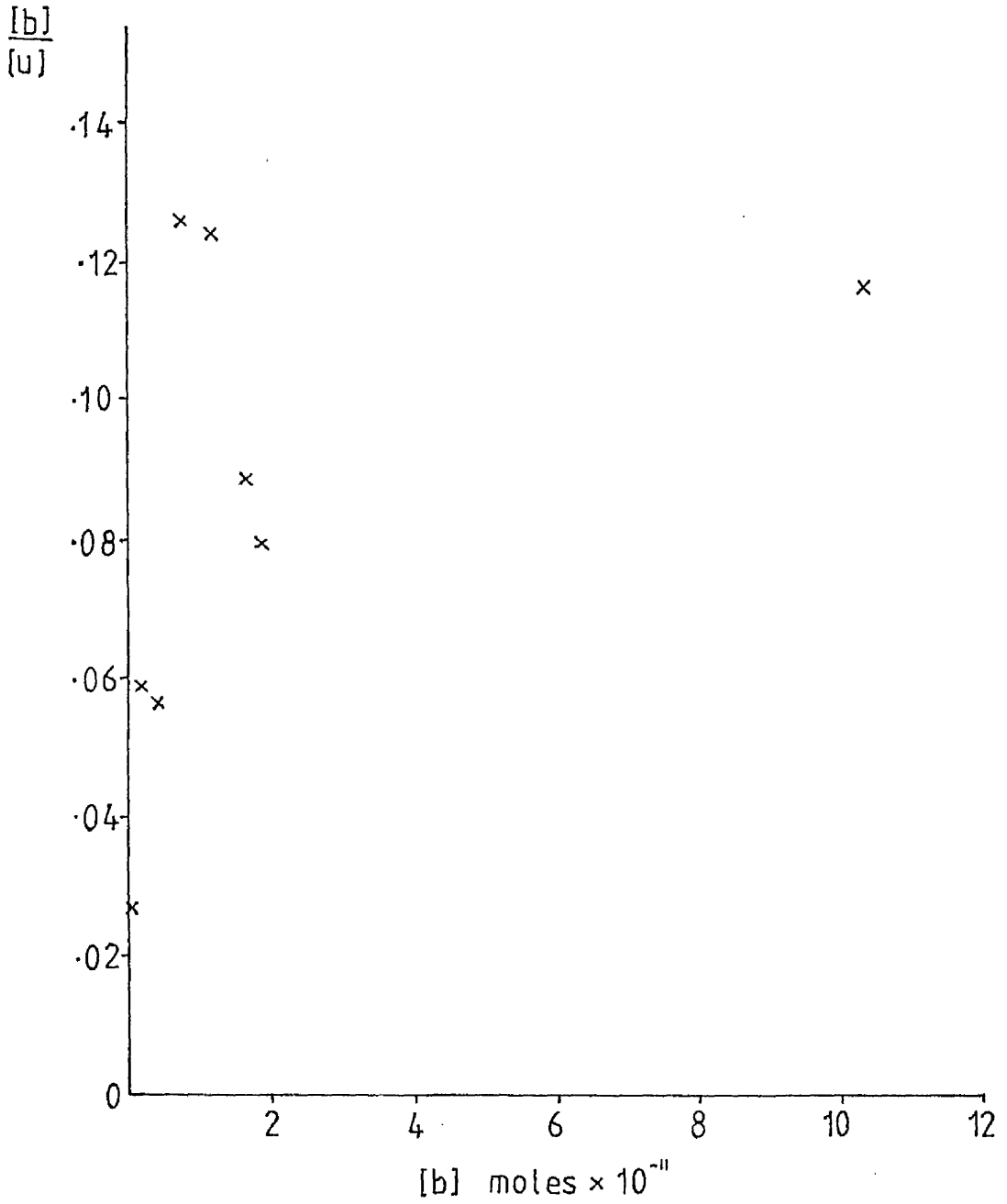


Fig. 36. Scatchard plot of results of progesterone-binding experiment using partially-purified AUP as source of receptor(s).

GENERAL DISCUSSION

Alpha uterine protein was first isolated from human amniotic fluid and subsequently detected in endometrium, myometrium, early gestation decidua, amnion and chorion by one-dimensional AACE (Sutcliffe et al, 1978). In this thesis several additional sources of AUP are reported, namely late gestation decidua, early gestation and term placentae and 17 week fetal gut. The protein known as "progestagen-dependent endometrial protein" (PEP), which has been shown to be identical to AUP, has also been reported in endometrium from both phases of the menstrual cycle, and early gestation decidua (Joshi et al, 1980a). The inability to detect PEP in term placenta (Joshi et al, 1980a) probably reflects the insensitivity of the assay techniques used, ie. immuno-electrophoresis and double immunodiffusion relative to AACE. Similarly, AUP may be present in other tissue and fluids in concentrations below the limits of detection by one-dimensional AACE. There is some evidence that this is the case: although AUP cannot be detected in maternal serum by immunodiffusion or one-dimensional AACE (Sutcliffe et al, 1978; Joshi et al, 1980a), low concentrations have been detected in this fluid by radioimmunoassay (R.G. Sutcliffe and colleagues, in preparation). Therefore, although the major sources of AUP are known, the complete tissue distribution remains to be established.

The available evidence argues strongly in favour of identity between AUP from the two major sources: uterine decidua and amniotic fluid, ie. decidual and amniotic fluid AUP are similar as regards molecular size, electrophoretic mobility on agarose gel and immunological cross-reactivity, and, from the studies on PEP, glycoprotein nature and isoelectric point. However, some anomalous findings are reported in this thesis. Firstly, there is an alteration in the normal

AUP precipitin peak morphology on AACE which is apparently a feature of antibody/antigen specificity in that it is only observed with antisera raised against decidual AUP. The cause of this altered peak morphology is not known, although it may indicate incomplete antigenic identity between AUP from these two sources. In addition, analysis of immune-precipitated ^{125}I-AUP (partially-purified from amniotic fluid) by SDS-polyacrylamide gel electrophoresis under denaturing conditions resulted in three bands of molecular weights 48,000, 16,000 and 9,000. This contradicts previous reports that AUP of decidual origin is a dimer of MW 50,000, composed of two similar or identical polypeptide chains (Sutcliffe et al, 1980; Joshi et al, 1980b). This may indicate that amniotic fluid AUP has a different subunit structure from that found in uterine decidua. However since these findings are unconfirmed it is impossible to discount alternative hypotheses that either some physical alteration of AUP occurred during the purification procedure or subsequent radio-iodination, or that the 16000 and 9000 molecular weight bands observed represent serum protein contaminants.

Second trimester amniotic fluid is readily-available due to the routine practice of amniocentesis, therefore appears to be an ideal source of AUP for use in a large scale purification of this protein. However, due to problems of a largely technical nature the attempt to purify AUP from amniotic fluid using the three-step method of antibody affinity chromatography, ion exchange chromatography and gel filtration described by Sutcliffe et al (1980) proved unsuccessful. The initial antibody affinity chromatography step resulted in a 39% recovery of AUP which is identical to the recovery of AUP from 11 week uterine decidua reported previously (Sutcliffe et al, 1980). In addition, there was considerable enrichment of AUP after this step together with a marked reduction in

protein content, ie. only 4% of the total protein applied to the affinity column was recovered in the eluted material (see Table 6). However, affinity chromatography was not entirely satisfactory as a step in the purification of AUP from amniotic fluid because the concentration of AUP is much lower in amniotic fluid than in 11 week decidua. Therefore in order to obtain measurable amounts of AUP it was necessary to apply a much larger volume of amniotic fluid as compared to decidual homogenate to the column. This in turn resulted in a higher degree of serum protein contamination when amniotic fluid was used as source material in the purification of AUP.

Ion exchange chromatography is a more suitable method than affinity chromatography for use in the purification of AUP from amniotic fluid because an ion exchange column has a greater protein carrying capacity per ml bed volume than an affinity column. Thus the problem of overloading the column is minimised. In addition, if the correct gradient of ionic strength is used, good separation of minor protein components can be achieved. Since the initial ion exchange step (during the three-step purification procedure) resulted in a good recovery of AUP and relatively good initial separation from contaminant proteins this was used as the first stage in the subsequent two-step purification procedure. Con-A Sepharose chromatography was used as the second stage because this is a highly-selective technique for the separation of glycoproteins. It was particularly useful in the purification of AUP from amniotic fluid because the quantitatively major protein contaminant, serum albumin, is not a glycoprotein hence does not bind specifically to Con-A. Although there was only a 20.5% recovery of AUP after this step (see Table 9), further enrichment of this protein occurred

and serum protein contamination was reduced. In particular, there was an approximately 12-fold reduction in the serum albumin concentration as compared to that in the material applied to the Con-A Sepharose column (see Fig 26). Therefore the two-step procedure of ion exchange chromatography and Con-A Sepharose chromatography resulted in partial purification of AUP with loss of the majority of the major contaminant, serum albumin. However, due to the low recovery of AUP from the Con-A Sepharose chromatography step further purification was not attempted.

Although the tissue distribution of AUP is fairly well-established, until recently there was little information on the actual site(s) of synthesis of this protein. As previously mentioned (see "Introduction") the consistent presence of AUP in both pregnant and non-pregnant uterine tissue suggested that this was the most likely site of synthesis. This has been confirmed by Joshi et al (1980a), who demonstrated synthesis of PEP in early gestation decidua, secretory phase endometrium and, to a much lesser extent, in proliferative phase endometrium. In addition, the exact cellular localisation of AUP within the endometrium has recently been established using an indirect immunoperoxidase technique. The results of this study indicate that AUP is present in the epithelial cells and secretions of the endometrial glands during the secretory phase of the menstrual cycle and during pregnancy (R.G. Sutcliffe and colleagues, in preparation). This clearly indicates that AUP is not only synthesised but also secreted by the progestational phase endometrium. The detection of AUP in placenta early in gestation (8-13 weeks) and at term by AACE suggests that AUP may be present in the placenta throughout gestation and that this may be an additional site of synthesis. Due to the close proximity of the placental trophoblast and decidua basalis after implantation,

however, it is possible that the AUP detected in placental tissue was in fact present in decidual cells adherent to the placenta. The data on the quantitation of AUP in early gestation (8-13 weeks) decidua and placenta argues against this possibility in that the concentration of AUP is very similar in the two tissues at each stage of gestation. Clearly, if placental AUP was synthesised by random associated cells of decidual origin the concentration of AUP would be reduced relative to that in decidua. In addition, in the immunoperoxidase study described above AUP was also found to be localised within the placental trophoblast, although staining was somewhat weaker than that observed in endometrium (R.G. Sutcliffe and colleagues, in preparation). AUP has also been detected in fetal gut by one-dimensional AACE, therefore this may be an additional site of AUP synthesis. Alternatively, the presence of AUP in the fetal gut may reflect swallowing of amniotic fluid by the fetus in utero, which is well-established by 17 weeks of gestation (Pritchard, 1965, 1966; Abramovich, 1970). The amnion and chorion may also be sites of AUP synthesis, but as yet there is no evidence to support this hypothesis.

The origin and possible routes of entry of AUP into the amniotic fluid have not yet been established. A decidual and/or placental origin appears most probable early in gestation, since secretion of AUP by the decidual glandular epithelium has been demonstrated and relatively high levels of AUP are found in both these tissues between 8 and 13 weeks of gestation, which precedes the period of high AUP concentration in amniotic fluid from 15-18 weeks (Sutcliffe et al, 1978). However, although there is an approximately ten-fold decline in the concentration of AUP in amniotic fluid between 12 weeks

of gestation and term, a much more considerable decline is apparent between early gestation and term in both decidua and placenta. This reduction in AUP concentration is to the order of 50- to 100-fold in decidua and 400- to 3000-fold in placenta. This may reflect either a dramatic decrease in the rate of synthesis of AUP with gestation in these tissues, or a less marked decline in synthesis together with increased secretion of AUP. Therefore, until these two possibilities are distinguished the origin of AUP in amniotic fluid late in gestation remains uncertain. As stated in the "Introduction", decidual AUP is more likely to enter the amniotic fluid via the "reflected" fetal membranes than by passage across the placental chorionic plate and associated amnion, because the chorionic plate is bathed with maternal blood. Clearly, AUP does not enter the amniotic fluid along with maternal serum proteins of similar molecular size because its concentration profile in amniotic fluid between 12 weeks of gestation and term differs from that of known maternal serum proteins (Queenan et al, 1970; Sutcliffe and Brock, 1972; Sutcliffe et al, 1978). In addition, only trace amounts of AUP are detectable in maternal serum (R.G. Sutcliffe and colleagues, in preparation). There are several possible routes by which placental AUP may enter the amniotic fluid. Firstly, AUP synthesised in the placental trophoblast may enter the surrounding maternal blood and cross the chorionic plate and amnion. For the reasons presented above AUP entering the amniotic fluid by this route is unlikely to contribute significantly to the amniotic fluid AUP pool. Secondly, AUP may enter the fetal blood vessels within the placenta and permeate into the amniotic fluid via the umbilical cord. Abramovich and Page (1972) demonstrated that the fetal skin and cord epithelium are permeable to fluid and some dissolved solutes up to mid-trimester.

Therefore it is possible that some exchange of proteins also occurs across the umbilical cord. The final and most likely route of entry of placental AUP into the amniotic fluid is by direct passage from the placental trophoblast across the chorionic plate and amnion. This, however, presupposes that an area of the placenta is not engulfed with maternal blood, but lies in close apposition to the chorionic plate.

An attempt to establish the origins in amniotic fluid of AUP, prolactin and α1,4-glucosidase by comparing levels of these proteins in 20 samples of second trimester amniotic fluid proved unsuccessful. The apparent lack of correlation between levels of AUP and α1,4-glucosidase may indicate that these two proteins have different origins in amniotic fluid. A correlation was previously found between levels of α1,4-glucosidase and heat-labile alkaline phosphatase between 13 and 18 weeks of gestation in separate samples of amniotic fluid (Sutcliffe et al, 1972b). This period of gestation corresponds to the early activity peak of heat-labile alkaline phosphatase in amniotic fluid. There is some evidence that the majority of this early activity may be due to the fetal intestinal type of this enzyme (Mulivor et al, 1979). Previous studies have indicated that the α1,4-glucosidase present in amniotic fluid and kidney is a different enzyme from the α1,4-glucosidase found in amniotic fluid cells, fibroblasts, leucocytes and liver which is deficient in Pompe's disease (Salafsky and Nadler, 1971a; Fluharty et al, 1973). However, fetal kidney is unlikely to be major source of α1,4-glucosidase in amniotic fluid (see "Introduction"). Therefore, characterisation of the type of α1,4-glucosidase present in decidua, fetal secretions and other fetal tissues, eg. skin, may resolve the question of the origin of this enzyme in amniotic fluid. Unfortunately,

the levels of amniotic fluid prolactin, which is almost certainly derived from the decidua and possibly the chorion could not be compared with the AUP and α1,4-glucosidase concentrations because of non-linearity of the radioimmunoassay results at different dilutions. As stated before, this may reflect alteration of the prolactin molecule caused by storage at -20°C. Thus, the observed nonlinearity may reflect measurement of a heterogeneous population of prolactin molecules which have different affinities for the anti-prolactin serum.

In contrast to the results for the probable non-serum proteins in the amniotic fluid samples, a strong positive correlation was observed between the three serum proteins measured: albumin, Gc and α_1-antitrypsin. As previously stated, Gc is a known maternal serum marker, hence this result is compatible with the proposed maternal serum origin of albumin and α_1-antitrypsin (Gitlin et al, 1964; Queenan et al, 1970; Sutcliffe and Brock, 1973; Johnson et al, 1974). If either albumin or α_1-antitrypsin originated from the fetus as has been suggested (Brzezinski et al, 1964; Kaiser et al, 1974), it is unlikely that such a relationship between their concentrations and that of Gc would have been evident. The fact that no correlation was observed between Gc and alpha fetoprotein, a fetal marker, supports this hypothesis. In addition, the molecular weight of α_1-antitrypsin is similar to that of Gc which freely permeates into the amniotic fluid from maternal serum, therefore it is unclear how a fetal α_1-antitrypsin deficiency as described by Kaiser et al (1974) could affect passage of maternal α_1-antitrypsin into the fluid.

The physiological function of AUP is not yet known. However, some suggestions of possible functions can be made with regard to the available information on tissue distribution, sites of synthesis and

concentration changes during gestation. From the distribution of AUP it is apparent that a pregnancy-associated function is most likely. However, synthesis of this protein has been demonstrated not only in decidual tissue but also in nonpregnant endometrium throughout the menstrual cycle (Joshi et al, 1980a). The rate of synthesis of AUP is higher in the decidua and secretory phase endometrium than in the proliferative phase endometrium, which may indicate that progesterone is involved in the synthesis and/or function of this protein. However, the results of the progesterone-binding study indicate that it is unlikely that AUP functions as a progesterone receptor. Although there is no direct evidence that progesterone has a role in the regulation of AUP synthesis, the increased rates of synthesis in the progestational-phase uterus suggest that this is likely.

Unfortunately, few studies have been carried out on human uterine-specific proteins during pregnancy. Most studies to date have involved the investigation of proteins present in the uterine fluid during the nonpregnant cycle. From such studies it is apparent that there is no quantitatively -dominant nonserum protein in the uterine secretions during the menstrual cycle. Also, the pattern of non-serum proteins is similar at both phases of the cycle (Maathuis and Aitken, 1978b). This is in direct contrast to the situation in most other mammals, in which there is a marked increase in uterine secretory activity during the pre-implantation (mid-luteal) period. In some cases this phase is characterised by the appearance of a dominant non-serum protein, eg. purple glycoprotein in the pig and uteroglobin in the rabbit (Aitken, 1979b). In humans, however, quantitatively minor components of the uterine fluid may have important functions. Alternatively, proteins with important pregnancy-associated functions may be synthesised

within the endometrium during the menstrual cycle but only released if fertilisation occurs. The stimulus for release of such proteins might be fertilisation or, more probably, implantation. In the case of AUP either of these two hypotheses may be correct since it is not known if this protein is present in the uterine fluid during the menstrual cycle. The possibility that the implanting blastocyst may stimulate uterine secretory activity in humans remains to be investigated. However, there is evidence that implantation causes a local increase in uterine secretory activity in the mouse, rabbit, wallaby and pig (Daniel, 1972; Renfree, 1972, 1973; Wyatt et al, 1976; Aitken, 1977b).

It has been suggested that AUP may have a role in the regulation of pre- or post-implantation embryonic development (Joshi et al, 1980b). Alternatively, AUP may be involved in the control of the actual implantation event. In humans, implantation is complete and the embryonic circulation established by about the 4th week after fertilisation (Corliss, 1976). Relatively high concentrations of AUP are present in the decidua and placenta between 8 and 13 weeks of gestation, but levels in these tissues before 8 weeks are not known. If secretion of AUP is induced by the implanting blastocyst and/or the AUP concentration is low during the first 8 weeks of gestation then a major role in the regulation of implantation or pre-implantation embryonic development is unlikely. Conversely, elevated levels of AUP during this period may indicate that such a role is possible. In either event, a regulatory role for AUP during the post-implantation period cannot be discounted. Other possible functions of AUP have been described already in the "Introduction". These include a minor role in fetal nutrition and the transportation of essential elements between the mother and fetus. Alpha uterine protein may serve either of these functions in vivo, although at present there is no evidence in support of this.

Conclusions and future objectives

The results presented in this thesis have contributed to some extent to our knowledge of the human protein known as alpha uterine protein or progestagen-dependent endometrial protein. However, there are several unresolved questions remaining and it is hoped that future research on this protein may elucidate these points. One such question is whether AUP from amniotic fluid is identical to that found in uterine decidua. Although AUP from these two sources appears to be biochemically and immunologically identical it has been shown that antisera raised against amniotic fluid and decidual AUP produce precipitin arcs of different morphology on AACE. In addition, amniotic fluid AUP may have a different subunit structure to uterine AUP. Therefore AUP from these two sources may be structurally dissimilar. Clearly, it is important to obtain purified amniotic fluid AUP in order to compare its structure with that of uterine AUP. The major problem associated with the purification of AUP from amniotic fluid is the relatively low concentration of this protein in the fluid. Therefore a successful purification technique must provide a means of enriching AUP without a concommitent increase in the concentration of the contaminating serum proteins, particularly serum albumin. Prior concentration of amniotic fluid (eg. by ultrafiltration) followed by specific removal of serum albumin by column chromatography may prove useful as a preliminary step in a future purification of AUP from amniotic fluid.

The discovery of AUP in placental tissue has not only contributed to data on the tissue distribution of this protein but also poses some interesting questions about the nature and function of AUP. This is particularly interesting in view of recent evidence that the AUP

detected in the placenta by AACE is present in the placental trophoblast cells, rather than in associated cells of decidual origin (R.G. Sutcliffe and colleagues, in preparation).

Although this protein has been termed "progestagen-dependent", there is as yet no direct evidence that progesterone is involved in the synthesis and/or function of AUP. However, increased synthesis of AUP in progestational-phase endometrium (Joshi et al, 1980a) suggests that this may be the case. AUP does not appear to be a progesterone-receptor, but it is possible that progesterone acting at its progesterone-receptor may thereby induce AUP synthesis. In vitro studies using cultured human endometrial cells, in which progesterone is added to the culture medium and levels of AUP in the cells and medium monitored, may resolve this question.

The major objective in any future study of AUP must be to establish the physiological function of this protein. Although AUP is synthesised in non-pregnant endometrium (Joshi et al, 1980a), it is not known whether it is secreted into the uterine fluid, or if secretion occurs only during pregnancy. From this, it may be deduced whether the function of AUP is associated solely with pregnancy. Therefore it is important to ascertain whether AUP is present in the uterine fluid during the normal menstrual cycle. Further information on the distribution and sites of synthesis of AUP may also aid in the elucidation of its function.

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